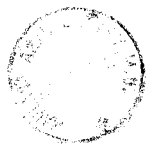
# PSORALEN AND ULTRAVIOLET LIGHT IN PIGMENT PRODUCTION

( SUMMARY )

A
THESIS SUBMITTED TO THE
ALIGARH MUSLIM UNIVERSITY
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN THE FACULTY OF SCIENCE

*by*
RASHID ALI, M. Sc.,

1965



# PSORALEN AND ULTRAVIOLET LIGHT IN PIGMENT PRODUCTION

A
THESIS SUBMITTED TO THE
ALIGARH MUSLIM UNIVERSITY
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN THE FACULTY OF SCIENCE

*by*
RASHID ALI, M. Sc.,

1965

## ACKNOWLEDGEMENT

The author wishes to express his gratitude to the Council of Scientific and Industrial Research, New Delhi, for the award of a Junior Research Fellowship and to Dr. M.L. Dhar, Director, Central Drug Research Institute, Lucknow, for permission to carry out this work and to submit this thesis.

He is deeply indebted to Dr. S.C. Agarwala, Assistant Director, Division of Biophysics, Central Drug Research Institute, Lucknow, for his constant interest and supervision throughout the course of these investigations.

He would further like to record his sincere thanks to Dr. A.R. Kidwai, Professor of Chemistry, Aligarh Muslim University, Aligarh, for encouragement and general supervision.

# C O N T E N T S


| | | Page No. |
|---|---|---|
| | PREFACE ... ... | 1 - 111 |
| CHAPTER I | GENERAL INTRODUCTION ... | 1 - 38 |
| | VITILIGO AND LEUCODERMA ... | 2 |
| | MELANIN PIGMENTATION ... | 5 |
| | CHEMICAL NATURE OF MELANIN | 6 |
| | BIOCHEMISTRY OF MELANIN FORMATION ... ... | 7 |
| | MELANOCYTE STIMULATING HORMONE ... ... | 10 |
| | INHIBITORS OF MELANIN FORMATION ... ... | 11 |
| | ROLE OF COPPER IN PIGMENTATION ... ... | 13 |
| | RADIANT ENERGY AND PIGMENTATION ... ... | 14 |
| | PSORALEN ... ... | 17 |
| | PHOTOSENSITIZING PROPERTIES OF PSORALENS ... ... | 18 |
| | PSORALEN THERAPY IN LEUCODERMA ... ... | 20 |
| | TOXICITY OF PSORALENS ... | 22 |
| | MODE OF ACTION OF PSORALENS | 24 |
| | REFERENCES ... ... | 25 |
| CHAPTER II | EFFECT OF SOLAR AND ULTRAVIOLET IRRADIATION ON PSORALEN ... ... | 39 - 59 |

| | | Page No. |
|---|---|---|
| CHAPTER III | INACTIVATION OF SULPHYDRYL GROUPS BY IRRADIATED PSORALEN ... ... | 60 - 83 |
| CHAPTER IV | PHOTO-OXIDATION OF DIHYDROXY-PHENYLALANINE (DOPA) IN PRESENCE OF PSORALEN ... | 84-102 |
| CHAPTER V | IN VIVO STUDIES IN RELATION TO GROWTH, ORGAN WEIGHT AND INCORPORATION OF $P^{32}$ IN ALBINO RATS ... ... | 103-114 |
| CHAPTER VI | IN VITRO AND IN VIVO EFFECT OF NORMAL AND IRRADIATED PSORALEN ON GLUCOSE METABOLISM OF BRAIN AND LIVER HOMOGENATE ... ... | 115-133 |
| CHAPTER VII | EFFECT OF FEEDING PSORALEN ON THE COPPER CONTENT OF DIFFERENT ORGANS IN ALBINO RATS ... ... | 134-151 |
| CHAPTER VIII | GENERAL SUMMARY AND CONCLUSIONS ... ... | 152-156 |


* * * * *

* * *

*

# PREFACE

"When one appreciates the concern felt by the white vitiligo patient, it is easy to understand the distress experienced by members of darker races in whom the pigmentary defect is more obvious. Small wonder then that when Prime Minister Nehru spoke before a group of drug manufacturers some years ago he expressed hope that advances would be forthcoming to help patients with the three most important diseases in India: tuberculosis, leprosy and vitiligo".

Lerner, A.B.

J.Invest.Dermatol.,
32 (1959) 235.

Vitiligo is an acquired progressive achromia caused by the loss of function of the tyrosinase system of the melanocytes at epidermo-dermal junction. Although the disease does not essentially interfere with the normal life expectancy of an individual, it may psychologically and socially disturb the whole pattern of his life. Studies on vitiligo have so far been approached from three directions: statistical, clinical and metabolic and

the most successful treatment evolved uptil now is the administration of furocoumarins orally, intraperitoneally or topically along with exposure to either ultraviolet or solar irradiation. A lot of work has been done on the clinical aspect of these drugs leading to certain definite conclusions. However, the metabolic aspect as well as its mechanism of action is largely unknown.

The present work has been undertaken to investigate a few aspects of the mode of action of psoralen <u>vis-a-vis</u> the effect of ultraviolet radiation thereon. The entire work in the present dissertation has been divided in eight chapters.

Chapter I on "General Introduction" deals with a brief description of vitiligo, its causes and the biochemistry of pigment production. The chemistry of psoralen and its role in the treatment of this disease has also been included.

The effect of ultraviolet and solar irradiation on psoralen forms the subject matter of second chapter. Separation of irradiated products has been done by paper chromatography and the ultraviolet absorption spectra of the eluates of different spots has been presented.

The third chapter is devoted to an study of the inactivation of -SH groups by irradiated products of psoralen. Succinic oxidase inactivation and the reversal of thiourea inhibition of potato tyrosinase has been taken as an index of inactivation of -SH groups.

Chapter IV deals with the stimulation of photo-oxidation of DOPA (dihydroxyphenylalanine) by psoralen, under the influence of ultraviolet, solar and white light. The influence of a few -SH containing compounds and ascorbic acid has been investigated **vis-a-vis** the stimulating effect of psoralen on the photo-oxidation of dopa.

Growth, organ weight and the incorporation of phosphorus as affected by prolonged (9 weeks) feeding of psoralen is presented in Chapter V. Chapter VI deals with **in vitro** and **in vivo** studies on glucose oxidation of brain and liver homogenates in rats.

The effect of feeding psoralen on the total copper content of different organs and tissues has been described in Chapter VII. Finally, the broad conclusions of this work have been summarized together under "General summary and Conclusions" in Chapter VIII.

* * *

# CHAPTER I

## GENERAL INTRODUCTION

In the ancient Indian Vedas[1-3] dating back to 1400 B.C. or even earlier, the cure of leprosy and leucoderma has been described with certain black seeds together with Bringaraga (Eclipta prostata), Indravaruni (colocynth) and Turmeric (Curcuma longa). On consultation of the classical Indian Medical Literature[4], it appears that the most widely used plant was Bavachee. Even in the Budhist literature[5] the cure of leucoderma with 'Vasuchika' (said to be the old form of Bavachee or (Psoralea Corylifolia) has been distinctly mentioned. Another plant Ammi majus, a weed found in Nile Valley has been employed for centuries as a cure for leucoderma[6]. Extensive researches on Psoralens may be said to have begun in 1941 in the laboratories of Fahmy and his group at the University of Cairo[7,8]. Confirmation of El-Mofty results were reported in France[9,10]. Clinical trials of this drug may be said to originate from the studies of Lerner, Denton and Fitzpatrick at the University of Michigan in 1952[11,12].

Active component from all these plants has been shown to be the furocoumarin type of compounds and the parent structure being that of psoralen. Before going into the details of furocoumarins, it would be advisable to have some idea of vitiligo as such and the general biochemistry of pigment production.

## VITILIGO AND LEUCODERMA

The word 'Vitiligo' is of Latin origin and means literally skin eruption. According to present concept[13] vitiligo refers to idiopathic depigmentation beginning soon after birth and is usually seen to be progressive. Leucoderma is a more general term and includes the hypopigmentation of vitiligo, albinism, burn or trauma, leprosy, syphilis and pinta. Depending on the type vitiligo has been divided by Lerner[13] as under:-

| Suggested terminology | Other terms |
|---|---|
| Vitiligo: Commonest form of vitiligo with the pigmentation of hands, face, axillas etc., part of which may be segmental in distribution. | Generalized vitiligo<br>Vitiligo vulgaris. |

| | |
|---|---|
| Complete vitiligo: Ordinary vitiligo frequently extends so that most of the body is involved. A few pigmented spots may remain. | Total vitiligo<br>Universal vitiligo. |
| Segmental vitiligo: Depigmentation segmental corresponding to nerve distribution, but some area within segment may be spared. | Loccolised vitiligo<br>Partial vitiligo<br>Focal vitiligo. |
| Perihalo nevus: Depigmentation surrounding a pigmented nevus. Later the nevus itself may become depigmented. | Leucoderma acquisitum<br>Sutton's disease. |

Vitiligo in general represents a common type of cutaneous depigmentation[13-19]. There may be varying degree of pigmentation in vitiligo with several shades of colour occuring in the same patient. Usually the normal skin is separated from the vitiligo region by a border which is hyperpigmented. The main portion of a vitiliginous patch may be completely depigmented or merely lighter in colour than the normal skin. It may extend over the entire body and the patient may have complete vitiligo.

The disease has been known to begin at any age but its onset is commonly found in younger adults. Usually the first lesions occur as depigmented spots on exposed area such as dorsal surface of the hands, the face and the neck. However the body folds, axilla and groin may also be the initial sites. The flexor

aspect of the arms tends to be more involved than the extensor. Facial lesions are common especially around the eyes and mouth. The eye lids are affected similarly. The other most common sites are genitals, periumbilical, perianal, inner aspects of thighs and knees, the anterior aspects of the legs etc. Graying of the hair is unpredictable, some patients with a limited vitiligo, may have a lot of gray hair, while many others with complete vitiligo have little grey hair.

Another point considered important in prognosis and treatment is the behaviour of the hair colour in vitiliginous patches[15]. In certain cases the hair retains it natural colour, even in large areas of long standing whereas in other cases the hair becomes depigmented sooner or later. Prognosis of repigmentation is better if the colour of the hair persists, because each hair may then serve as a centre from where pigmentation may spread. In rare cases white hair have been observed to be repigmented under treatment. The pigmentory systems of epidermis and hair thus seem to be under independent control.

Lerner[30] has further reported that under resting condition, more sweat occurs in areas of vitiligo and that vitiliginous skin does not become yellow during

Jaundice or on ingestion of atabrine. Electrical resistance has also been reported to be decreased in vitiligo. A metabolic study performed on 25 patients with vitiligo[13], disclosed no change in urinary noradrenaline and melanocyte stimulating hormone. Serum copper, liver or thyroid function tests or skin temperature were also seen to be normal. In one study Agarwala et al.[21] found the urinary 17-Ketosteroid excretion to be low in different groups of vitiligo patients. Histamine[22] and -SH groups[23] have on the other hand been reported to be present in normal amounts.

## MELANIN PIGMENTATION

Robin[24] is credited for having the first to have used the term 'melanin'. Heusinger[25] examined the skin of the Negroes and identified a brown pigment in the stratum corneum. He also studied localised melanosis in liver, the skin of pregnant women, brown moles in typhus and scruvy, and has demonstrated yellow, brown and black stuff in mucosae. During his work on the cytology of skin Henle[26] observed a pigment layer between the epidermis and dermis over the entire body of the Negroes. Ehrmann[27,28] has

reported a detailed account of the pigment cells in areola, skin of the genitalia, nape and hips of women. Pigmentation of the Negro fetus was studied by Zimmermann et al.[29], who found melanocytes in the third month of intrauterine life, and thereafter the dopa reaction was found to be strongly positive.

## CHEMICAL NATURE OF MELANIN

'Melanin' is usually considered to be derived from the Greek word 'Melas' meaning black[30]. According to Mason[31] melanin is a pigment of high molecular weight which occurs either uncombined or as conjugates with protein and is formed enzymatically by the oxidation of phenols. Thomas[32] restricts the term melanin to black nitrogenous pigments. Its designation includes the various shades of brown and black pigments found in mammals, insects, plants and marine animals. Melanin can be decolourised from jet black to light tan by sodium hydrogensulphite[33,34] or ascorbic acid[35]. This decolourization can be reversed by the addition of potassium ferricyanide. The pigment is mostly insoluble in organic solvents. An approximate composition of melanin[36,37] from

natural and synthetic sources is as follows:-

| | |
|---|---|
| Carbon | 57% |
| Hydrogen | 3½% |
| Nitrogen | 9% |

Oxygen is also present, and in some natural melanins, varying amount of sulphur has also been reported. In some cases it has been possible to make the pigment free of sulphur[38]. Use of electron spin resonance spectroscopy has in addition given evidence of the free radical property of melanins[39-44]. Recently their infra-red spectra[45] has been obtained and indication of exact chemical structure have also been published[46].

## BIOCHEMISTRY OF MELANIN FORMATION

The enzymatic steps involved in melanin formation have been most successfully investigated in plants and funci. In 1895 Bourquelot and Bertrand[47] reported the presence a substance in mushroom, Russula nigricans, which was capable of being converted into a black pigment. This substance was latter shown to be tyrosine, and the enzyme in the fungus was named as tyrosinase[48]. Bloch and Ryhiner[49]

reported that frozen sections of a fresh human skin when placed in a 1:1000 aqueous solution of L-3,4, dihydroxyphenylalanine (dopa) at pH 7.4, developed blackening of the dendritic cells at epidermo-dermal junction. Inspite of the fact that tyrosinase was found to be widely distributed in nature (its existence was reported in numerous plants, bacteria, invertebrate and vertebrate) contradictory reports appeared for a long time about the presence of tyrosinase in melanin forming mammalian tissues[50-53]. The confusion was once for all cleared by the studies of Hogeboom and Adams[54] who found transplantable mouse melanoma to contain tyrosinase. The properties of tyrosinase[55,56] and its histochemical demonstration have subsequently been described[57-62].

Tyrosinase is one of a large group of copper containing oxidases found within intracytoplasmic structures called melanin or pigment granules. The granules for a long time have been thought to originate in mitochondria, since their sedimentation from homogenised mouse melanomas, was always associated with the usual mitochondrial enzymes[63-64]. However, recent electron-microscopic studies of human hair bulb melanocytes conclusively established structural differences between melanin granules and mitochondria within the same cells[65-68]. Recently subcellular

localization of melanin biosynthesis have been described by Seiji et al.[69].

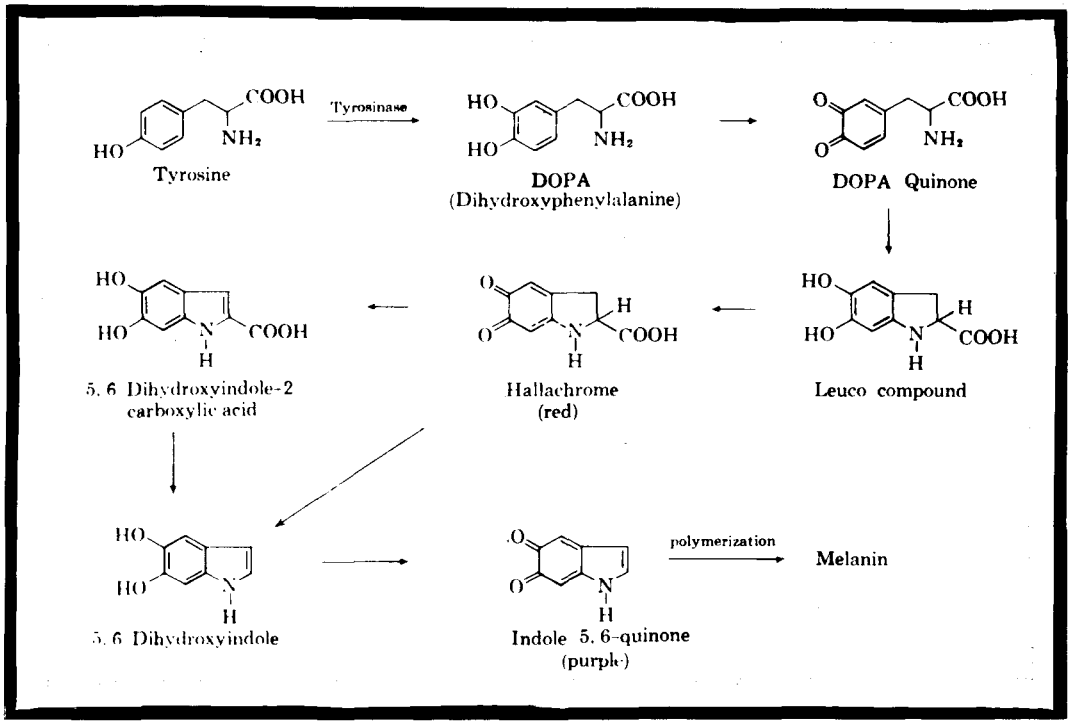
Structurally melanin is formed by the coupling of the quinonoid polymer, 5,6-indole with a protein, the polymer in itself being formed from tyrosine by the action of aerobic tyrosinase, as shown in Fig.1[70-72,30].

The various factors that have been described to determine the rate and amount of melanin formation include[30].

1. The availability of the melanin precursor, tyrosine.
2. The rate of tyrosinase synthesis.
3. The presence of factors that activate tyrosinase (e.g. dopa).
4. The presence of naturally occuring inhibitors of the tyrosine-melanin pathway (-SH groups).

Conversion of tyrosine, but not of dopa, to melanin is characterised by variable lag period, which becomes prolonged in the case of low concentrations of tyrosinase. It had long been considered by several authors including Rothman that tyrosine was probably the mother substance of melanin since dopa could not be demonstrated in the human skin. The

FIG.1


Tyrosinase
Tyrosine
DOPA
(Dihydroxyphenylalanine)
DOPA Quinone
Leuco compound
Hallachrome
(red)
5, 6 Dihydroxyindole-2
carboxylic acid
5, 6 Dihydroxyindole
Indole 5, 6-quinone
(purple)
polymerization
Melanin

concept was confirmed in the form of tyrosine reaction perfected by Fitzpatrick et al.[57].

## MELANOCYTE STIMULATING HORMONE

Removal of pituitary has been found to result in a permanent lightning of the skin colour, which could be restored by injecting pituitary extract. Lee and Lerner[73,74] and others[75,76] announced the isolation of two melanocyte stimulating hormones, $\alpha$ and $\beta$-MSH, from hog pituitary gland. Their structures revealed them to be polypeptide in nature which were smaller but related to ACTH[77-80]. MSH from cow differs from that of hog only in one respect, the second amino acid of the polypeptide chain of bovine MSH is serine instead of glutamic acid[79]. Administration of MSH is found to produce rapid darkening of the skin and is alleged to cause formation of pigmented nevi[81-85]. When MSH is added to a pigment cell, melanin granules are seen to clump about the nucleus, streaming out towards the periphery and finally becoming uniformaly distributed making the melanocytes opaque. The possible mechanism of action of MSH have also been described[86].

It is known that other hormones such as adrenaline, noradrenaline, acetylcholine, serotonin and triidothyronine can make the melanocytes lighter in colour. The lightning agent, usually designated as melatonin, was isolated from pineal gland and has the structure of 5-hydroxyindole[87-92]. In addition to human pineal gland it has been demonstrated in bovine hypothalamus and peripheral nerves[89]. In frog skin melatonin acts as the most potent known lightning agent. It reverses the process of MSH, the granules stream back towards the centre of the cell and the major portion appears transparent. Modification of Thyroid activity by melatonin have also been described[93]. Adrenaline causes clumping and decreases movement of pigment granules in melanocytes from mouse melanomas and normal skin[94].

## INHIBITORS OF MELANIN FORMATION

Among the inhibitors of tyrosine-tyrosinase reaction, those compounds which inactivate the copper component of the enzyme have been much more extensively studied and are of physiologically greater importance[95]. Such inhibition can usually be achieved *in vitro* by binding or removing the copper ions, necessary for tyrosinase action, common

inhibitors of this type usually are organic sulphur compounds[30,54,96-98], hydrogen sulfide, carbon monoxide and cyanide ions. Some of the organic sulphur compounds containing reactive sulfhydryl groups are diethyldithiocarbamate, cysteine, glutathione, 2,3-dithiopropanol (BAL), thiourea and its derivative such as phenylthiourea, allylthiourea, $\alpha$-napthylthiourea and thiouracil. Carbon monoxide and cyanide ions form strong covalent bonds with copper and thereby bring about the inhibition of tyrosine-tyrosinase activity.

Increased pigmentation is frequently observed when heavy metals such as arsenic, bismuth, iron, gold, silver and mercury are deposited in the skin, and most probably these metals bind the epidermal sulfhydryl groups and thereby activating tyrosinase[99]. Since -SH groups have been considered to be important <u>in vivo</u> determinants of tyrosinase action. However, if larger amounts of these metals are present, they are capable of replacing the copper of tyrosinase giving rise to inactive enzyme and consequent depigmentation.

In the actual process of melanin formation dopa is first oxidised to dopa quinone, which is then further oxidised. If dopa quinone is removed from

reaction, melanin production is considerably inhibited. Aminophenyl compounds such as aniline, 3-aminotyrosine, and p-phenylenediamine have been seen to combine with o-quinones and are therefore potential inhibitors of melanin formation[100]. Substances e.g. ascorbic acid which can bring about the reduction of o-quinone, can also inhibit the pigment production[101].

Some derivatives of tyrosine as N-acetyltyrosine, N-formyltyrosine, 3-fluorotyrosine are also known to be effective inhibitors of melanin formation[102].

## ROLE OF COPPER IN PIGMENTATION

In view of the importance of copper as the prosthetic group of polyphenol oxidases, its role in tyrosine-tyrosinase action and thereby pigment production is of special significance[103]. A diet deficient in copper invariably results in depigmentation in a variety of animals e.g. rats, cats, rabits and cattle[104-107]. Addition of trace amounts of the copper salts to the deficient diet has been observed to restore pigmentation.

The valence state of the <u>in vivo</u> copper is largely unknown. However, phenolase isolated in cupric form is reported to be inactive[108]. For

proper functioning the mammalian tyrosinase requires its reduction from dopa to form an active cuprous enzyme[109]. Mason[110] proposed a hypothesis for mechanism of action of tyrosinase (Fig.2). Recently Bright et al.[111] have discussed an ionic mechanism for its action.

## RADIANT ENERGY AND PIGMENTATION

Therapy of vitiligo by ultraviolet irradiation seems to have been initiated by D.W. Montgomery[112] in 1940, who used a Finsen lamp on a Mexican boy aged 19. The boy was given nine exposures of ten minutes each and the affected patches were seen to turn red or sunburnt within four months. However, the face was reported to be repigmented and the spots on the hands had almost disappeared.

The melanin response to sunlight or ultraviolet is generally considered to occur in three stages as given below[70,113,114]:-

(a) Melanin darkening,

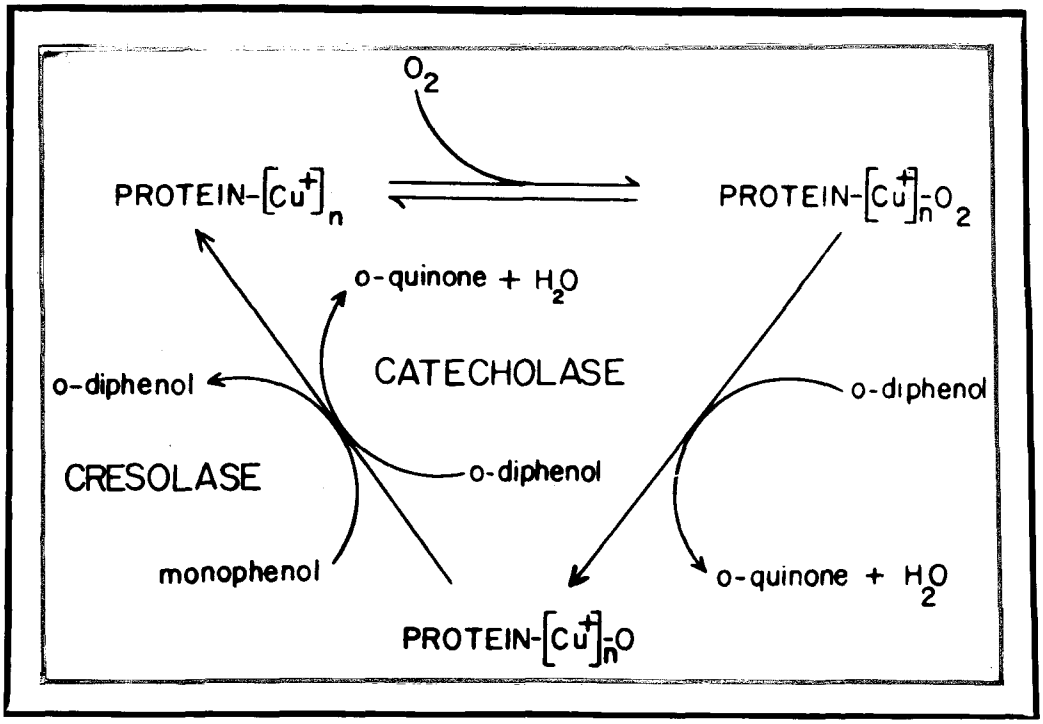
(b) Melanin migration and

(c) Melanin formation.

The melanin darkening is observed within a few minutes after exposure to relatively longer wavelength

Fig. 2 Role of copper in tyrosinase action.

FIG. 2


O2
PROTEIN-[Cu+]n
PROTEIN-[Cu+]n-O2
o-quinone + H2O
CATECHOLASE
o-diphenol
o-diphenol
CRESOLASE
o-diphenol
monophenol
o-quinone + H2O
PROTEIN-[Cu+]n-O

(3000-4200A°) with a maximally effective action spectrum of 3400A°[115]. Miescher[116] reported erythema by rays of wavelengths under 3200A°. Following sunlight or ultraviolet exposure, a marked increase in the level of tyrosinase activity has been seen in epidermal melanocytes[57,117]. Even after several days of exposure to solar or ultraviolet radiation melanocytes are seen to be in a state of hyperpigmentation.

Melanin formation following exposure to ultraviolet is mostly considered to be the result of increased melanogenesis in existing melanocytes rather than melanocyte proliferation. According to the reflectance studies of Edwards and Duntley[118] melanin formation begins after two days, reaches a maximum after 19 days and ceases after one month. The skin does not return to its initial melanin content however until nine and a months later. Blum[119] is of opinion that ultraviolet exposure gives rise to a thickening of the stratum corneum as a protective measure rather than the increase of melanin. This tolerance to subsequent ultraviolet exposure is possible because of the greater scattering which occurs in the stratum corneum. Thomson[120] on the other hand, holds the view that melanin in the stratum corneum is responsible for the difference between negro and white skin rather than difference

in its thickness. Hamperl et al.[121] explained the action of ultraviolet on the basis of a photochemical splitting of nucleic acid in the nucleus, which may be responsible for the erythema as an inflammatory reaction. Rothman concept of the effect of ultraviolet irradiation is mainly the inactivation of inhibitors, chiefly -SH compounds in the skin, thereby releasing the tyrosinase and giving rise to better pigmentation.

In view of the above findings it may be said that the exact biochemical mechanism, underlying this activation by solar or ultraviolet irradiation is not clearly understood. As mentioned in the preceding paragraph some of the hypothesis advanced for their action are as under[122-127]:-

1. Trace amount of dopa is formed by irradiation of tyrosine solution which may be responsible for the activation of tyrosinase.
2. Level of -SH groups is reduced which brings about an enhancement of tyrosine-tyrosinase action.
3. Decrease in redox potential, and thus favouring tyrosinase activity.
4. Resultant erythema may elevate temperature and favour melanin formation. However melanin formation is known to occur with air or water cooled

ultraviolet when in there is hardly any elevation of skin temperature.

## PSORALEN

The furocoumarin group of compounds includes psoralen, 8-methoxypsoralen (8-MOP) and other related compounds[128]. These compounds have been seen to inhibit the growth of certain plants without otherwise harming them[129-131]. They have also been reported to be effective antifungal agents[132]. Their wide spread occurrence in nature and chemistry have been reviewed on several occassions[133-136].

The absorption spectra of furocoumarins usally consists of three absorption maxima one at 325 mμ or less, the second between 230-270 mμ and the last at 290-330mμ[137]. These absorption maxima have been reported[11] to change as a result of irradiation in the case of 8-MOP and a generalized absorption is obtained which gets stronger towards shorter wavelengths. With psoralen two new compounds, namely the dimer and furocoumaric acid have been reported after irradiation. Somewhat similar results have been reported with 8-methoxypsoralen and 5-methoxypsoralen.

PHOTOSENSITIZING PROPERTIES OF PSORALEN

Psoralen photosensitization, in several biological systems, has been reported by a number of investigators[138-145]. Some of the more common examples are:-

(a)

Topical application of psoralens on human and other mammalian skin, exhibits photosensitized erythemal response skin to sunburn, generally followed by increased cutaneous pigmentation and thickening of stratum corneum.

(b)

The lethal action on several bacteria is significantly increased.

(c)

Enzymes such as succinic dehydrogenase, lactic dehydrogenase and cytochrome oxidase are more readily inactivated and

(d)

Seed germination in some cases has been reported to be considerably inhibited.

In all cases of psoralen photosensitization oxygen is not normally required. As regards the mechanism of this photosensitization, Pathak et al.[146,147] reported that furocoumarins exhibit fluorescence and undergo electronic transition involving Singlet to Triplet inter combinations, which may lead to free radical formation, and eventual biological changes in the irradiated system.

Among the different furocoumarin analogues and their derivatives the parent compound psoralen has been shown to exhibit maximum photodynamic activity[148]. In decreasing order of activity the compound are psoralen, 4,5', 8-trimethylpsoralen, 4-methylpsoralen, 5'8-dimethylpsoralen 8-methoxypsoralen, 5-methoxypsoralen, 4',5'-dihydroxanthotoxin, psoralen glucoside and 8-isoamyleneoxypsoralen.

From the above order it would be seen that methoxy substitution on 5 or 8 positions decreased the activity, and replacement with hydroxy, nitro, amino groups rendered the compound inactive. The active sites in the psoralen molecule for photosensitization are thus generally said to be:-

1. Valence bonds between carbon 3 and 4 and between 5 and 8.

2. Intact lactone ring.
3. Fusion of furan and coumarin ring at carbon 6 and 7.
4. Unsaturated bond between carbon 4' and 5'.

## PSORALEN THERAPY IN LEUCODERMA

Therapeutic use of furocoumarins in vitiligo may be said to have originated from the work of El-Mofty[8] in 1952. In 1953 Lerner et al.[11] reported an increased ability to tan after oral intake of 8-MOP followed by exposure to sunlight. Fitzpatrick et al.[12] and subsequently Arnold[149] and Becker[150] reported essentially the same type of results and their study confirmed the previous reports with regards to methoxsalen.

An extensive study on 50 test patients in the Arizona state prison was reported by Fitzpatrick et al.[12]. Administration of 30 mg of methoxsalen orally and subsequent exposure to sunlight produced multiple effects including erythema, edema and pigmentation. The pigmentation was seen to be evident even after one year, in some patients. These studies led to the conclusion that methoxsalen had no protective effect but on the contrary potentiated several of the skin responses to ultraviolet light[151].

Elliot[152] observed that 67% of the patients felt that the results obtained justified the cost of the treatment and the time expended. However cent per cent pigmentation could only be achieved if treatment was continued over periods long with sufficient intensity. It was demonstrated[153] in eight normal subjects that a 30 minutes exposure to sunlight, two hours after ingesting 30 mg of methoxsalen could increase the tolerance of the exposed skin to subsequent exposures to ultraviolet. In another 25 normal persons[154] methoxsalen taken orally before exposure to sunlight provided protection against sunburn in 23 cases and produced increased pigmentation in 18 persons.

The response of 8-MOP, taken orally has only been seen in patients who tend to be practical and take a specified dose of the drug, prior to sun exposure. By careful manipulation of the therapeutic dose and the subsequent solar exposure it is possible in many persons to greatly increase the skin pigmentary responses to sunlight with hardly any increase in the skin inflammation[155].

The amount of repigmentation[156] in general seemed to depend more on the total time of exposure to sunlight and the location of the lesions than

on the age of the patient, duration of the disease or the extent of involvement. Coloured children are seen to have a good response with a more rapid and yet complete onset of benefit.

The histological and clinical changes produced by psoralen and its allied compounds[157] have been observed to be so similar that they probably represent a pattern more often seen with any photosensitizer. None of these photosensitizers produced a change which ultraviolet alone could not produce but the reaction was markedly accentuated.

## TOXICITY OF PSORALENS

Contradictory reports have appeared in the literature regarding the toxicity of psoralens[154-156,158]. London[156] observed toxic reactions of mild to moderate degree in oral methoxsalen therapy. The toxic symptoms were severe nausea and vomiting, nervousness, blistering and dermatitis. Stegmaier[154] while studying the clinical aspect of 8-MOP found that one patient developed a basal cell carcinoma of skin, whereas another lost muscular coordination. Yet in another case severe cheilitis and marked edema of the feet was seen to develop. Toxic effects in general have

been known to include severe nausea and vomiting, acute nervousness and depression. Liver function tests carried out by different investigators[159-161] have indicated mostly normal limits. Lerner et al.[11] and Elvi[162] reported liver necrosis after the administration of psoralen. Mukherji[163] has shown that feeding of psoralen and imperatorin, and a mixture of psoralen and isopsoralen to albino rats resulted in 20 per cent increase in the weight of liver.

Griffin and his collaborators[164,165] have further shown that administration of 8-MOP alongwith ultraviolet exposure resulted in the formation of tumors of ears and face in mice. O'Neal and Griffin[166] also reported that 8-MOP actually decreased the carcinogenic effect of ultraviolet radiations when fed to mice, but when it was administered intraperitoneally one hour before exposure, the carcinogenic effect of radiation was found to be increased and latent period was reduced. Pathak et al.[167] could however observe no protection against ultraviolet carcinogenesis by dietry feeding of psoralen or 8-MOP. Hakim et al.[168] on the other hand have observed tumors of ears in mice of varying degree of pigmentation when administered with methoxsalen either in diet or when injected intraperitoneally, with simultaneous exposure to fluorescent light.

## MODE OF ACTION OF PSORALENS

Inspite of a large number of clinical investigations on psoralens a great deal of confusion still exists regarding their mechanism of action. It has been shown that systematic administration of appropriate doses of methoxsalen followed by ultraviolet exposure produces a series of morphological changes in skin[157,159]. Among the general explanations regarding the mode of action of psoralens is based on the hypothesis that the altered epidermis simply increases retention of melanin formed in ordinary amounts in response to solar irradiation. The second suggestion involves the stimulation of melanocytes giving rise to increased production of melanin. Some of the mechanisms for 8-MOP action proposed by Judis[170] are as under:-

(a) It photo-oxidizes certain inhibitors of melanin formation present in abnormal amounts in vitiliginous area.

(b) 8-MOP may photo-oxidize the available dopa to melanin.

(c) It may somehow raise the oxidation reduction potential in the system making conditions more favourable for normal melanin formation.

The purpose of this dissertation therefore is to describe certain aspects of the mode of action of psoralen, an outline of which has already been presented in the beginning as a preface.

REFERENCES


1. Bloomfield, M., 'Sacred Books of East', Hymns of Atharva-Veda, Vol.XLII, Clarendon Press, Oxford, 1897.

2. Whitney, W.D., 'Atharva-Veda Samhita' (translation and notes), Harvard Oriental series, Vol.7, Lanman, Harvard University Press, Cambridge, Mass. 1905.

3. Fitzpatrick, T.B. and Pathak, M.A., J.Invest.Dermatol., 32 (1959) 229.

4. Jolly, J., Medicine K.J.Trubner, Strassburg, 1901.

5. Hoernle, A.F.R., 'Studies in Medicine of Ancient India', Bower Manuscript, Calcutta, Government Printing, India, 1893-1912.

6. Ibn El-Baitar, Mofradat El-Adviya 11,4 (Cited by Fahmy, I.R., Quart.J.Pharm.Pharmacol., 20 (1947) 291).

7. Fahmy, I.R. and Abu-Shady, H., Quart.J.Pharm.Pharmacol., 20 (1947) 291.

8. El-Mofty, A.M., J.Roy.Egypt.Med.Assoc., 31 (1948) 697.

9. Sidi, E. and Bourgeois-Gavardin, J., — J.Invest.Dermatol., 18 (1952) 391.

10. Sidi, E., Bourgeois-Spinasse, J. and Planat, P., — Dyschromies et vitiligo, Expansion Scientifique Francaise, Paris, 1957.

11. Lerner, A.B., Denton, C.R. and Fitzpatrick, T.B., — J.Invest.Dermatol., 20 (1953) 299.

12. Fitzpatrick, T.B., Hopkins, C.E., Blickenstaff, D.D. and Swift, S., — Ibid., 25 (1955) 187.

13. Lerner, A.B., — J.Invest.Dermatol., 32 (1959) 285.

14. Idem — 'Vitiligo', Transactions, Pacific Dermatologic Association, 7th Annual Meeting, Mexico City, 1955 pp.25-38.

15. Pinkus, H., — J.Invest.Dermatol., 32 (1959) 281.

16. Kugelman, T.P. and Lerner, A.B., — J.Biol.Med., 33 (1961) 407.

17. Khoo, O.T., — Singapore Med.J., 3 (1962) 157.

18. Cordero, C.F.A., — Rev.Col.Med.Guatemala, 12 (1961) 183.

19. Mostafa, M.V., — Medicus (Karachi), 24 (1962) 57.

20. Lerner, A.B., — Am.J.Med., 19 (1955) 902.

21. Agarwala, S.C., Mukerji, B. and Sharma, R., — J.Endocrinol., 13 (1956) 195.

22. Quiroga, M.I., Guillot, C.P. and Manjou, F., — Rev.Argent.Dermatosifil., 33 (1949) 17.

23. Lajmanovich, S. and Magnin, P.H., — J.Invest.Dermatol., 25 (1955) 79.

24. Robin, C.P., — 'Anatomie et Physiologie Cellulaire', Paris, J.B. Bailliere et Fils, 1873.

25. Heusinger, K.F., — 'Untersuchungen ueber die Anomalie Kohlen und Pigmentbildung in dem menschlichen Korper'. In Eisenach: System der Histologie, J.F.Baerecke, 1823.

26. Henle, — Allgemeine Anatomie,1943.

27. Ehrmann, S., — Vierteljahrschr.Dermatol. Syph., 17 (1885) 507.

28. Idem — Ibid., 18 (1886) 57.

29. Zimmermann, A.A. and Cornbleet, T., — J.Invest.Dermatol., 11 (1948) 383.

30. Lerner, A.B. and Fitzpatrick, T.B., — Physiol.Rev., 30 (1950) 91.

31. Mason, H.S., — 'Structure of Melanins' In Pigment cell Biology, M.Gordon, Ed. Academic Press, New York, N.Y., 1959, p.353.

32. Thomas, M., — 'Melanins' In Modern methods of Plant Analysis. K.Paech and M.V.Tracey,Eds.IV Springer, Berlin,Germany, (1955) p.661.

33. Figge, F.H.J., — Proc.Soc.Exp.Biol.Med., 41 (1939) 127.

34. Idem — Ibid., 44 (1940) 393.

35. Rothman, S., — J.Invest.Dermatol., 5 (1942) 67.

36. Kastle, J.H., — 'The oxidases and other Oxygen Catalysts concerned in Biological Oxidations', U.S. Hygienic Laboratory Bulletin (No.59), Washington,D.C., Government Printing Office, 1910.

37. Wells, H.G., — 'Chemical Pathology',Being a discussion of General Pathology from the stand point of the chemical process involved (5th Ed.) Philadelphia, W.B.Saunder Company, 1925 pp.527-528.

38. Schaaf, F., — Biochem.Z., 209 (1929) 209.

39. Commoner, B., Townsend, J. and Pake, G.E., — Nature, 174 (1954) 689.

40. Edwards, M.L., Kerkut, G.A., Munday, K.A., Ingram, D.J.E., Saxena, M.C. and Leach, K., — Ibid., 184 (1959) 1402.

41. Edwards, M.L., Kerkut, G.A., Munday, K.A. and Leach, K., — Ibid., 190 (1961) 1005.

42. Mason, H.S., Ingram, D.J.E. and Allen, B., — Arch.Biochem.Biophys., 86 (1960) 225.

43. Longuet-Higgins, H.C., — Ibid., 86 (1960) 231.

44. Cope, F.W., Sever, R.F. and Polis, B.D., — Ibid., 100 (1963) 171.

45. Bonner, T.G. and Duncan, A., — Nature, 134 (1962) 1078.

46. Swan, G.A., — Ann.N.Y.Acad.Sci., 100 (1963) 1005.

47. Bourquelot, E. and Bertrand, G., — Compt.Rend.Soc.Biol., 47 (1895) 532.

48. Bertrand, G., — Compt.Rend.Acad.Sci., 122 (1896) 1215.

49. Bloch, B. and Ryhiner, — 'Histochemische Studien in Nebenlebenden Gewebe Ueberfermentive Oxydation und Pigmentbildung' Ztschr. f.d.ges. Exp.Medizin, 1917).

50. Durham, F.M., — Proc.Roy.Soc.London, Ser.B, 74 (1904-5) 310.

51. Pugh, C.E.M., — Biochem.J., 27 (1933) 475.

52. Onslow, H., — Proc.Roy.Soc.London, Ser.B, 89 (1917) 36.

53. Russel, W.L., — Genetics, 24 (1939) 645.

54. Hogeboom, G.H. and Adams, M.H., — J.Biol.Chem., 145 (1942) 273.

55. Kazuo Shimao, — Biochim.Biophys.Acta, 62 (1962) 205.

56. Brown, F.C. and Ward, D.N., — J.Biol.Chem., 233 (1958) 77.

57. Fitzpatrick, T.B., Becker, S.W.(Jr), Lerner, A.B. and Montgomery, H., — Science, 112 (1950) 223.

58. Kutika, A. and Fitzpatrick, T.B., — Ibid.,121 (1955) 893.

59. Morris Foster, — Proc.Soc.Exp.Biol.Med., 29 (1932) 713.

60. Kugelman, T.P. and Van Scott, E.J., — J.Invest.Dermatol., 37 (1961) 73.

61. Morris Foster and Robinson Cook,(Jr), — Proc.Soc.Exp.Biol.Med., 85 (1954) 120.

62. Szabo, G., — Arch.Dermatol., 76 (1957) 324.

63. Lerner, A.B., Fitzpatrick, T.B., Calkins, C.E. and Summerson, W.H., — J.Biol.Chem., 178 (1949) 185.

64. Du Buy, H.G., Woods, M.W., Burk, D. and Lackey, M.D., — J.Nat.Cancer.Inst., 9 (1949) 325.

65. Birbeck, M.S.C., Mercer, E.H. and Barnicot, N.A., — Exp.Cell Res., 10 (1956) 505.

66. Birbeck, M.S.C. and Barnicot, N.A., — 'Electron microscope studies on pigment formation in human hair follicles'. In Pigment cell Biology, M.Gordon, Ed. Academic Press, New York, N.Y. (1959) pp.549-561.

67. Baker, R.V., Birbeck, M.S.C., Blaschko, H., Fitzpatrick, T.B. and Seiji, M., — Nature, 187 (1960) 392.

68. Seiji, M., Fitzpatrick, T.B. and Birbeck, M.S.C., — J.Invest.Dermatol., 36 (1961) 243.

69. Seiji, M., Shimao, K., Birbeck, M.S.C. and Fitzpatrick, T.B., — Ann.N.Y.Acad.Sci., 100(1963) 497.

70. Fitzpatrick, T.B. and Szabo, G., — J.Invest.Dermatol., 32 (1959) 197.

71. Lerner, A.B. and Case, J.D., — Ibid., 32 (1959) 211.

72. Kenney, J.A.(Jr), J.Nat.Med.Assoc.,N.Y., 53 (1961) 447.

73. Lerner, A.B. and Lee, T.H., J.Am.Chem.Soc., 77 (1955) 1066.

74. Lee, T.H. and Lerner, A.B., J.Biol.Chem., 221 (1956) 943.

75. Porath, J., Roos, P., Landgrebe, F.W. and Mitchell, G.M., Biochim.Biophys.Acta, 17 (1955) 599.

76. Benfey, B.J. and Purvis, J.L., J.Am.Chem.Soc., 77 (1955) 5167.

77. Harris, J.I. and Roos, P., Nature, 173 (1956) 90.

78. Geschwind, I.I., Li, C.H. and Barnafi, L., J.Am.Chem.Soc., 79 (1957) 4404.

79. Idem Ibid., 79 (1957) 6304.

80. Harris, J.I. and Lerner, A.B., Nature, 179 (1957) 1346.

81. Snell, R.S., J.Endocrinol., 25 (1962) 249.

82. Lerner, A.B., and McGuire, J.S., Nature, 189 (1961) 176.

83. McGuire, J.S. and Lerner, A.B., Ann.N.Y.Acad.Sci., 100 (1963) 622.

84. McGuinness, B.W., Ibid., 100 (1963) 640.

85. Lee, T.H., Lerner, A.B. and Buettner-Janusch, V., Ibid., 100 (1963) 652.

86. Lerner, A.B., Lee, T.H., Wright, M.R. and McGuire, J.S., Acta Endocrinol., 34 (1960) 73.

87. Lerner, A.B., Case, J.D., Takahashi, Y., Lee, T.H. and Mori, W., — J.Am.Chem.Soc., 80 (1958) 2587.

88. Lerner, A.B., Case, J.D., Mori, W. and Wright, M.R., — Nature, 183 (1959) 1821.

89. Lerner, A.B., Case, J.D. and Heinzelman, R.V., — J.Am.Chem.Soc., 81 (1959) 6084.

90. Lerner, A.B., Case, J.D. and Takahashi, Y., — J.Biol.Chem., 235 (1960) 1992.

91. Lerner, A.B. and Case, J.D., — Federation Proc., 19 (1960) 590.

92. Ippen, H., — Deut.Med.Wochschri., 86 (1961) 307.

93. Raschieri, L., Du Luca, F., Cramarossa, L., De Martino, C., Oliverio, A. and Negri, M., — Experientia, 19 (1963) 15.

94. Stubblefield, E., Escue, R.B. and Uff, J., — Science, 126 (1957) 29.

95. Lorincz, A.L., — 'Pigmentation' In physiology and Biochemistry of skin, Rothman, S., Ed. The University of Chicago Press, Chicago (1954), pp.515-563.

96. Paschkis, K.E., Cantarow, A., Hart, W.M. and Rakoff, A.E., — Proc.Soc.Exp.Biol.Med., 57 (1944) 37.

97. DuBois, K.P. and Ervay, W.P., — J.Biol.Chem., 165 (1946) 711.

98. Bernheim, F. and Bernheim, M.L.C., J.Biol.Chem., 145 (1942) 213.

99. Ormsby, O.S. and Montgomery, H., 'Disease of the skin' 8th Edition Philadelphia, Lea and Febiger, 1954 p. 721.

100. Pugh, C.E.M. and Raper, H.S., Biochem.J., 21 (1927) 1370.

101. Miller, W.H., Mallette, M.F., Roth, L.J. and Dawson, C.R., J.Am.Chem.Soc., 66 (1944) 514.

102. Lerner, A.B., Fitzpatrick, T.B. and Summerson, W.H., Federation Proc., 8 (1949) 218.

103. Peter Flesch, Proc.Soc.Exp.Biol.Med., 70 (1949) 79.

104. Keil, H.L. and Nelson, V.E., J.Biol.Chem., 93 (1931) 49.

105. Carter, F.J., Nature, 136 (1935) 195.

106. Sjollema, B., Biochem.Z., 235 (1931) 372.

107. Smith, S.W. and Ellis, G.H., Arch.Biochem., 15 (1947) 81.

108. Kubowitz, F., Biochem.Z., 292 (1937) 221.

109. Lerner, A.B., Fitzpatrick, T.B., Calkins, C.E. and Summerson, W.H., J.Biol.Chem., 178 (1950) 799.

110. Mason, H.S., 'Mechanism of Oxygen Metabolism', Advances in Enzymology. 12 (1957) 134. Interscience Publishers, New York, N.Y.

111. Bright, H.J., Wood, B.J.B. and Ingraham, L.L., — Ann.N.Y.Acad.Sci., 100 (1963) 985.

112. Montgomery, D.W., — J.Cutan.Dis., 22 (1904) 17.

113. Pathak, M.A., Riley, F.J., Fitzpatrick, T.B. and Curven, W.L., — Nature, 193 (1962) 149.

114. Pathak, M.A., Riley, F.J. and Fitzpatrick, T.B., — J.Invest.Dermatol., 39 (1962) 435.

115. Fitzpatrick, T.B., Pathak, M.A., Magnus, I.A. and Curven, W.L., — Ann.Rev.Med., 14 (1963) 195.

116. Miescher, G., — Arch.Dermatol.Syph., 180 (1940) 239.

117. Hanke, W., — Strahlentherapie, 115 (1961) 134.

118. Edwards, E.A. and Duntley, S.Q., — Am.J.Anat., 65 (1939) 1.

119. Blum, H.F., — Physiol.Rev., 25 (1945) 483.

120. Thomson, M., — J.Physiol., 127 (1955) 236.

121. Hamperl, H., Henschke, U. and Schulze, R., — Arch.Path.Anat., 304 (1939) 19.

122. Snell, R.S., — J.Invest.Dermatol., 40 (1963) 137.

123. Idem — Ibid., 40 (1963) 233.

124. Freeman, R.G., Cockerell, E.G., Armstrong, J. and Knox, J.M., — Ibid., 39 (1962) 295.

125. Mitchell, F.E., — Ibid., 41 (1963) 199.

126. Knox, J.M., — J.Chronic Diseases, 13 (1961) 391.

127. Quevedo, W.C.(Jr), — J.Soc.Cosmetic Chemists, 14 (1963) 609.

128. Fowlks, W.L., — J.Invest.Dermatol., 32 (1959) 249.

129. Bennett, E.L. and Bonner, J., — Am.J.Botany, 40 (1953) 99.

130. Musajo, L., — Il Farmaco.Pavia Ed.Sci., 10 (1955) 2.

131. Rodighiero, G., — Giorn.Biochim., 3 (1954) 139.

132. Chakraborty, D.P., DasGupta, A. and Bose, P.K., — Ann.Biochem.Exp.Med. (Calcutta), 12 (1957) 57.

133. Dean, F.M., — Prog.in the chem.of Organic Natural Products, 2 (1952) 225.

134. Sethna, S.M. and Shah, N.M., — Chem.Rev., 36 (1945) 1.

135. Spath, E. and Kaffner, F., — Monatsh., 52 (1936) 75.

136. Soine, T.O., — J.Pharm.Sci., 53 (1964) 231.

137. Nakabayashi, T., Tokoroyama, T., Miyazaki, H. and Isono, S., — J.Pharm.Soc.Japan, 73 (1953) 669.

138. Fowlks, W.L., — J.Invest.Dermatol., 32 (1959) 233.

139. Fowlks, W.L., Griffith, D.G. and Oginsky, E.L., — Nature, 181 (1959) 571.

140. Oginsky, E.L., Green, G.S., Griffith, D.G. and Fowlks, W.L., — J.Bacteriol., 78 (1959) 821.

141. Pathak, M.A. and Fellman, J.H., — Proc.Intern.Congr. Photobiology, 1960, Elsevier Publishing Company, Amsterdam, 1961, p.552.

142. Clark, J., — J.Invest.Dermatol., 37 (1961) 171.

143. Musajo, L. and Rodighiero, G., — Experientia, 19 (1963) 153.

144. Pathak, M.A., Fellman, J.H. and Kaufman, K.D., — J.Invest.Dermatol., 35 (1960) 165.

145. Bonner, J., — 'Personal communication' cited by Fowlks, W.L. 128.

146. Pathak, M.A., — J.Invest.Dermatol., 37 (1961) 377.

147. Pathak, M.A., Allen, B., Ingram, D.J.E. and Fellman, J.H., — Biochim.Biophys.Acta, 54 (1961) 503.

148. Pathak, M.A. and Fitzpatrick, T.B., — J.Invest.Dermatol., 32 (1959) 255.

149. Arnold, H.L.(Jr), — Hawaii Med.J., 16 (1957) 391.

150. Becker, S.W.(Jr), — 'Effect of 8-MOP and sunlight on Pigmentation and skin structure'. Presented at the fourth cosmetic seminar, Sept.,1957.

151. Daniels, F.(Jr), Hopkins, C.E., Imbrie, J.D., Bergeron, L., Miller, O., Crowe, F., and Fitzpatrick, T.B., — J.Invest.Dermatol., 32 (1959) 321.

152. Elliott, J.A.(Jr), — Ibid., 32 (1959) 311.

153. Imbrie, J.D., Farrington, D.(Jr), Bergeron, L., Hopkins, C.E. and Fitzpatrick, T.B., — J.Invest.Dermatol., 32 (1959) 331.

154. Stegmaier, O.C., — Ibid., 32 (1959) 345.

155. Kanof, N.B., — Ibid., 32 (1959) 343.

156. London, I.D., — Ibid., 32 (1959) 315.

157. Becker, S.W.(Jr), — Ibid., 32 (1959) 263.

158. Tucker, H.A., — Ibid., 32 (1959) 277.

159. Fitzpatrick, T.B., Labby, D. and Imbrie, J.D., — J.Am.Med.Assoc., 167 (1958) 1598.

160. Labby, D.H., Imbrie, J.D. and Fitzpatrick, T.B., — J.Invest.Dermatol., 32 (1959) 273.

161. Mukherjee, B.B. and Mukherjee, S.K., — J.Sci.Indus.Res.(India), 20C (1961) 326.

162. Elwi, A.M., — J.Egypt.Med.Assoc., 33 (1950) 773.

163. Mukherji, A., — J.Sci.Indus.Res.(India), 19C (1960) 273.

164. Griffin, A.C., — J.Invest.Dermatol., 32 (1959) 367.

165. Griffin, A.C., O'Neal, M.A., and Fitzpatrick, T.B., — Congr.Inter.Biochim. Resumes Comms. 3e Congr., Brussels, 1955 p.121.

166. O'Neal, M.A. and Griffin, A.C., — Cancer Research, 17 (1957) 911.

167. Pathak, M.A., Daniels, F., Hopkins, C.E. and Fitzpatrick, T.B., — Nature, 183 (1959) 799.

168. Hakim, F.E., Griffin, A.C. and Knox, J.M., — Arch.Dermatol., 82 (1960) 572.

169. Zumerman, M.C., — J.Invest.Dermatol., 32 (1959) 269.

170. Joseph Judis, — J.Am.Pharm.Assoc., 49 (1960) 447.

# CHAPTER II

## EFFECT OF SOLAR AND ULTRAVIOLET IRRADIATION ON PSORALEN

Ultraviolet light for a long time has been known to be highly effective in bringing about stimulation of melanin formation in human skin[1-3]. Following sunlight or ultraviolet exposure there is a marked increase in the level of tyrosinase activity[4] in epidermal melanocytes and hence melanin formation. The exact biochemical mechanisms, underlying this activation are not clearly understood. However, either trace amounts of dopa formed by irradiation of tyrosine solution may activate tyrosinase, or level of -SH groups may be reduced at the active site by an enhancement of tyrosine-tyrosinase reaction[5,6]. The enhancement of melanin formation as brought about by solar or ultraviolet irradiation may be said to involve two separate photobiological phenamenon[7]. The first designated as "Primary melanization" is brought about by erythemal spectrum (wavelengths shorter than 320 mμ with peaks at 250 and 297 mμ). It consists of an erythemal response followed by the formation of new pigment and migration of melanin granules. The second process namely "pigment darkening" is evoked by wavelengths longer than those which induce sunburn with maximum

effect at 340 mμ. Pigment darkening is actually known to begin immediately after exposure to ultraviolet, without any latent period.

The effect of ultraviolet light[8], on the melanocytes and melanin in the skin of guinea pigs, revealed stimulated melanogenesis, as shown by the increase in the amount of melanin in melanocytes, with commensurate increase in their size. They in addition have been found to have a larger, wider and more complex dendritic processes and there was a great increase in the amount of free melanin. The population density of the melanocytes hence was reported to rise considerably as a result of the treatment. Freeman et al[9]. studied the effect of sunlight on the thickness of the epidermis. Knox[10] has presented a detailed discussion on the effect of sunlight on human skin. He showed that most cutaneous changes from sunlight are caused by long wavelength ultraviolet light. The diseases in which sunlight is a causative or contributory factor includes sunburn and suntan, actinic degeneration, carcinogenesis and photosensitive dermatoses. The effect of X-rays[11] on melanocytes and melanin in the skin of the anterior abdominal wall was studied in six pure black and six pure red male guinea pigs. The treatment resulted in a considerable increase in the amount of melanin present, both inside and outside the melanocytes. This was accompanied by an enhancement of the cell size and increase in the

length, width and complexity of the dendritic process. There was also a marked increase in the melanocyte count.

Further potentiations of these ultraviolet effect has been observed by the in vivo administration of certain furocoumarins[13-14] e.g. psoralen and 8-methoxy-psoralen (8-MOP), but the actual mechanism by which this effect is produced is not clearly understood. Administration of furocoumarins and subsequent irradiation with either ultraviolet or solar light has been found to produce changes in the skin, such as thickening, increased density and increased adherence of the stratum corneum and the formation of a structure resembling stratum lucidum[15-17]. However furocoumarins have been reported to be inactive by themselves unless aided by ultraviolet light.

Lerner[18] reported alteration in the absorption spectra of 8-MOP as a result of irradiation, the change consisting of a loss of the characteristic peaks of the spectrum and a generalized absorption getting stronger towards shorter wavelengths. Upon irradiation of psoralen Fowlks[19] noted the same response and chromatography of the resulting solution revealed at least two new fluorescent compounds. One of these was designated as dimer and the other was found to be furocoumaric acid, identical with the one reported by Stoll[20]. Similar results were obtained with irradiation of 3 and 5-MOP. A new photochemical reaction between furocoumarin and

flavin mononucleotide (FMN) was reported by Musajo et al[21]. When aqueous ethanolic solution of equimolecular mixtures of FMN and furocoumarins (Psoralen, Xanthotoxin and Bergapten) were irradiated with a ultraviolet lamp of principal wavelengths at 3655A°, new spots were detected after chromatography which did not appear when FMN and furocoumarins were irradiated separately or when FMN was replaced by FAD.

It was therefore considered of interest to investigate the action of solar and ultraviolet irradiation on psoralen under different conditions and in the presence of compounds containing -SH groups. The results of these studies are reported in the present chapter. The ultraviolet absorption spectra of psoralen and some of the products obtained after irradiation has also been included.

## METHODS AND MATERIALS

### Ultraviolet irradiation of psoralen

Suspensions of finely powdered psoralen (3 mg in 3 ml water or buffer) were irradiated with a ultraviolet lamp (Honovia chromatolite 30, maximum emission at 2537A°) kept at a distance of 9-10 cm from the surface of suspension. The sample was placed over a magnetic stirrer in order to have a uniform distribu-

tion during irradiation. In one experiment irradiation of psoralen was also done with $\beta$-rays of $P^{32}$ as ortho-phosphate. The petri dish was covered with tin foil on the sidesto avoid radiation loss.

Solar irradiation of psoralen

Psoralen was dissolved in alcohol (1:1) giving a concentration of 1 mg/ml. Ten ml of the solution was taken in a 50 ml glass stoppered cylinder, which was kept under bright sun in horizontal position. After the requisite period, the cylinder was removed and kept in dark in a refrigerator until used.

Chromatography of irradiated psoralen

In view of the difficulties experienced by earlier workers[22-26] in chromatographic separation of furocoumarins and the nonresolution and diffuseness of bands on unbuffered Whatman No.1 paper strips, separation in the present studies were carried out in the descending manner on paper strips buffered with 0.1M $Na_2HPO_4$, using organic phase of n-butanol saturated with water (4:1) as the developing solvent. 0.2 ml to 0.3 ml of the irradiated psoralen solution was applied on chromatographic paper. In the case of ultraviolet irradiated psoralen the supernatant solution after ultraviolet irradiation of psoralen or other mixtures were applied as bands, while in the case of solar

irradiated psoralen, the total amount as such was applied. The bands were applied with an Agla Micrometer syringe fitted on to a capillary pipette so as to deliver small amounts at a time, and to keep the strip narrow. For larger volumes multiple application technique was followed. The first band was allowed to dry completely before second application. The strips were then allowed to saturate in the chamber for at least four hours. The solvent was then added and was allowed to flow overnight, after which the paper was removed and dried in air. The chromatograms were visualized under ultraviolet and the flourscent bands were carefully marked.

Ultraviolet spectra

The fluorescent area for a particular compound was carefully taken out by the help of clean scissors. Corresponding area from the control paper was also taken out for comparison. The compounds were eluted with small volume of 1:3 ethanol and after proper dilution, the ultraviolet absorption spectra was determined in a Beckmann DU spectrophotometer, from 200 mμ to 400 mμ. The ultraviolet absorption spectra of psoralen per se and the irradiated mixture was also observed for proper evaluation.

RESULTS

Effect of ultraviolet irradiation on psoralen

The $R_f$ values of the products obtained after irradiation of psoralen under different conditions are recorded in Table I. It is seen that under the present conditions of irradiation, psoralen gave rise to at least five products having different $R_f$ values. The major product obtained on irradiation, either in water suspension or aqueous ethanol or as band, had $R_f$ values of 0.63, 0.64 and 0.76 respectively. The absorption spectrum (Fig.1) of the eluate of this product indicated a generalized absorption, getting stronger towards the shorter wavelength region. No distinct maxima were obtained between 220 and 390 mμ, as against the usual three maxima observed with psoralen. From the absorption spectra this compound appeared to be different from that of dimer and furocoumaric acid as reported by Fowlks. The absorption spectrum of aqueous supernatant of irradiated psoralen as a whole was however, similar to that of psoralen. The data recorded in Table I would also show that irradiation of psoralen in the presence of an aqueous solution of cystaine hydrochloride produced a number of fluorescent products having $R_f$ values of 0.04, 0.13, 0.41, 0.59 and 0.67, besides that of psoralen ($R_f$ 0.94). The psoralen control eluted from buffered paper had, as usual, maxima

Table I - $R_f$ values of products produced by ultraviolet irradiation of psoralen under different conditions.

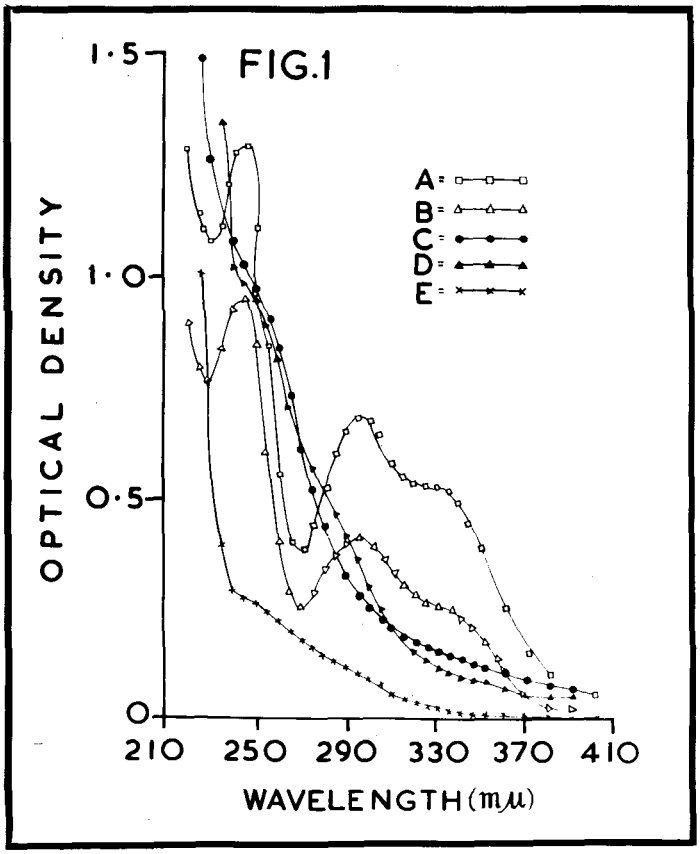
(Irradiation in all experiments was for 6 hours at 37°C).

| Forms in which psoralen was irradiated | Mean $R_f$ values of fluorescent products | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Ethanol:water suspension (2:1 or 1:1) | 0.00* (++) | - | - | 0.44 (+) | - | 0.63 (+++) | - | 0.83 (++) | 0.93 (+++) |
| Water suspension | 0.00 | - | - | 0.43 | - | 0.64 | - | 0.84 | 0.93 |
| As a band on buffered paper | 0.00 (+++) | 0.19 (+) | - | 0.41 (++) | - | - | 0.76 (++) | - | 0.92 (++) |
| 0.1M phosphate buffer (pH7.4) suspension | 0.03 (++) | - | - | - | 0.58 (++) | - | - | - | 0.92 (++) |
| Dilute HCl suspension | - | - | - | - | 0.60 (++) | - | - | 0.83 (+) | 0.93 (+++) |
| 0.1M Phosphate buffer (pH8.5) suspension | 0.00 (+) | - | - | 0.41 (+) | 0.60 (++) | - | - | - | 0.93 (++) |
| Water suspension+ cysteine HCl (1mg/ml) | 0.04 (++) | 0.19 (+) | - | 0.41 (+) | 0.59 (++) | 0.67 (+++) | - | - | 0.94 (++) |
| Water suspension+ cysteine HCl (1mg/ml) buffered, pH 7.4 | 0.03 (+) | - | - | 0.42 (++) | 0.60 (++) | - | - | - | 0.94 (++) |
| Water suspension+ glutathione (1mg/ml) | 0.01 (+) | 0.16 (+) | 0.28 (+++) | 0.45 (+) | 0.58 (+++) | - | 0.71 (+++) | - | 0.93 (++) |
| Water suspension+ $Na_2S_2O_4$ (1mg/ml) | 0.03 (+) | - | 0.37 (+) | 0.45 (+) | 0.54 (+++) | 0.62 (+++) | - | 0.81 (+) | 0.93 (++) |

*The intensity of fluorescent bands given in parenthesis is indicated as follows: +, faint; ++, medium; +++, strong.

Fig. 1 Ultraviolet absorption spectra of (A) supernatant from irradiant aqueous suspension of psoralen; (B) psoralen eluted with water from a chromatogram; (C) water eluate of intense fluorescent band ($R_f$ 0.63); irradiation of psoralen was done in ethanol-water mixture; (D) ethanol (95 per cent) eluate of band ($R_f$ 0.67) of irradiated psoralen and cysteine hydrochloride; and (E) ethanol (95 per cent) eluate of band ($R_f$ 0.67) of incubated psoralen and cysteine hydrochloride (60°C. for 6 hr) mixture.

FIG.1


1·5
1·0
0·5
0
OPTICAL DENSITY
A
B
C
D
E
210
250
290
330
370
410
WAVELENGTH (mμ)

at 245, 290 and 330 mμ. The absorption spectra of the fluorescent products likewise had no maxima between 220 and 330 mμ and showed a generalized absorption which became stronger towards shorter wavelength region.

Incubation of psoralen with aqueous cysteine hydrochloride (1 mg/ml) for 24 hours or longer at 37°C (pH of resulting solution 2-3) was also found to produce a number of fluorescent compounds with $R_f$ values 0.022, 0.20, 0.53 and 0.67, besides psoralen band ($R_f$ 0.94). The formation of these products was found to depend on pH, time and temperature of incubation. Incubation of psoralen with aqueous cysteine hydrochloride at 25°C for 4 hours did not produce these products; incubation for 24 hours, however, produced only one compound with $R_f$ value 0.67. The other products could be formed in a shorter time by incubation at 60°C and also by increasing the amount of added cysteine hydrochloride.

Incubation of psoralen with glutathione (1 mg/ml) at 25°C for 24 hours was also found to produce the band with $R_f$ value 0.66 along with the band for psoralen. The ultraviolet absorption spectrum of the eluate of compound with $R_f$ values 0.67 produced by incubating psoralen with aqueous cysteine hydrochloride also showed the same pattern of absorption as described earlier, for other fluorescent products. Psoralen incubated in a solution of dilute hydrochloric acid at the same pH as that of cysteine hydrochloride in phosphate buffer

(pH 7.4 or higher) did not produce any of these products. That these products are not the reduction products of psoralen was demonstrated by incubating psoralen with aqueous sodium hydrosulphite solution for 72 hours, when none of the above products were formed. Irradiation of psoralen, however in the presence of aqueous sodium hydrosulphite, brought about the formation of a number of fluorescent bands whose $R_f$ values are given in Table I. Irradiation of psoralen with $\beta$-rays from $P^{32}$ orthophosphate or with 100 Watt electric bulb did not produce any new products.

Effect of solar irradiation on psoralen

The $R_f$ values of the different products obtained after solar irradiation of psoralen, under different conditions, are recorded in Table II. Even irradiation for 30 minutes gave rise to products having different $R_f$ values. The major products formed under the different conditions appears to be one giving bluish fluorescence ($R_f$ 0.36) and the other having greenish bright fluorescence ($R_f$ 0.43). The intensity of these spots (as seen by enhanced fluorescence) increased with the time of irradiation unto about two hours after which no further increase was seen. The other products obtained were minor in nature showing weak fluorescence. After longer exposures amounting to six hours newer fluorescent products have also been obtained which have

Table II - $R_f$ values of products obtained after solar irradiation of psoralen* for different periods.

| Irradiation period (hours) | Mean $R_f$ of fluorescent products | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 0.5 | 0.015 (+)** | - | - | 0.28 (+) | 0.35 (+) | 0.57 (+) | 0.88 (+++) |
| 1.0 | 0.012 (++) | - | - | 0.26 (++) | 0.34 (+++) | 0.57 (++) | 0.90 (+++) |
| 2.0 | 0.014 (++) | 0.06 (+) | - | 0.27 (+++) | 0.35 (+++) | 0.57 (++) | 0.86 (++) |
| 4.0 | 0.010 (++) | 0.06 (+) | 0.14 (+) | 0.26 (+++) | 0.33 (+++) | 0.54 (++) | 0.83 (++) |
| 6.0 | 0.013 (++) | 0.07 (+) | 0.12 (+) | 0.26 (+++) | 0.35 (+++) | 0.53 (++) | 0.90 (++) |

*Psoralen in water:alcohol (1:1) was irradiated.

**The intensity of fluorescent bands given in parenthesis is indicated as follows: +, faint; ++, medium, +++, strong.

been included in Table II. The absorption spectra of the psoralen irradiated for six and twelve hours have been presented in Fig. 2 and 3. As is evident from the figures, irradiation upto six hours did not bring about any change in the pattern of spectra however with psoralen irradiated for twelve hours the peaks of the spectra became less prominent, as compared to control.

The absorption spectra of eluates of spots having $R_f$ values of 0.26, 0.34 and 0.57 indicated a generalized absorption getting stronger towards the shorter wavelength region, essentially different from that of dimer or furocoumaric acid as reported by Fowlks, but similar to the ones reported earlier in this chapter, when psoralen was irradiated with ultraviolet light. In the absorption spectra of all these products, no distinct maxima were obtained between 230 and 420 mμ, although the eluate of psoralen ($R_f$ 0.90) showed the usual three maxima at 245, 290 and 330 mμ[27].

The data recorded in Table III show that irradiation of psoralen in the presence of an aqueous solution of cysteine hydrochloride produces a number of fluorescent products having $R_f$ values of 0.033, 0.122 0.17, 0.33 and 0.53 besides that of psoralen ($R_f$ 0.95). The psoralen control eluate from buffered paper had maxima at 245, 290 and 330 mμ. The absorption spectra of the fluorescent products likewise had no maxima

Ultraviolet absorption spectra of six hours (Fig.2) and twelve hours (Fig.3) solar irradiated psoralen.

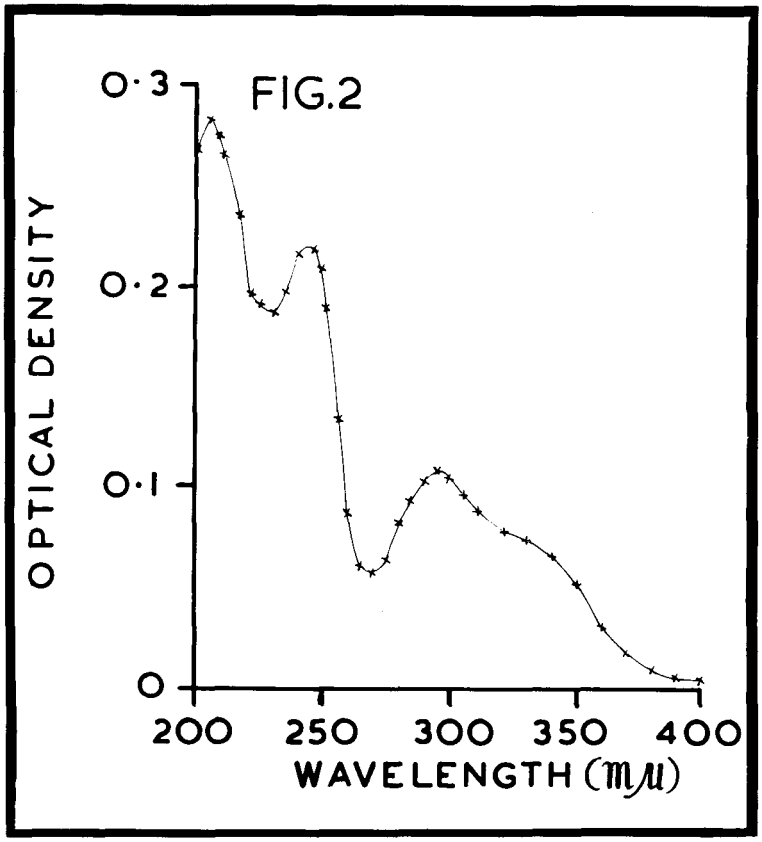


FIG.2
0·3
0·2
0·1
0
OPTICAL DENSITY
200
250
300
350
400
WAVELENGTH (mμ)

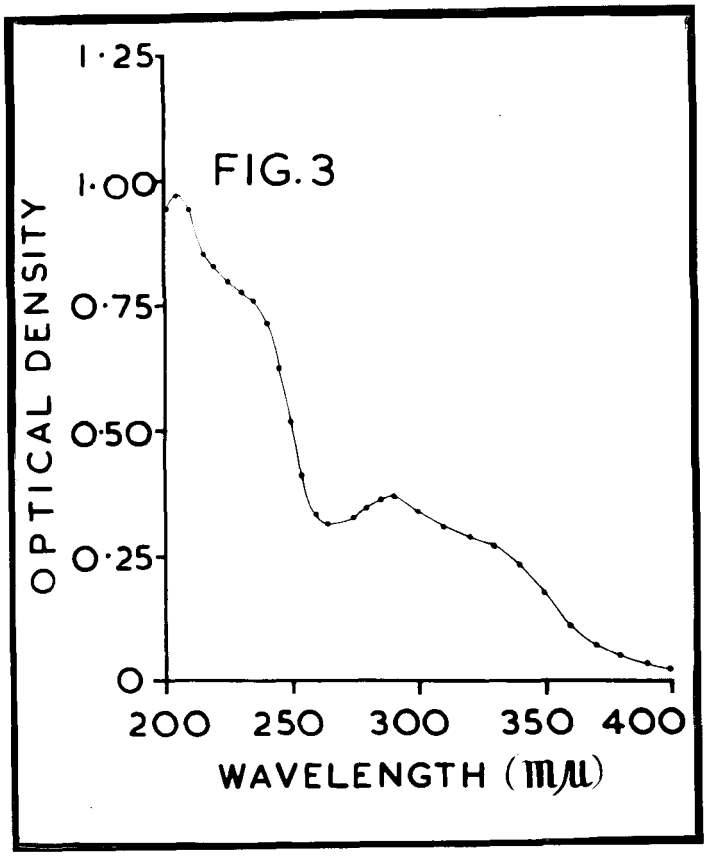


FIG.3
1·25
1·00
0·75
0·50
0·25
0
OPTICAL DENSITY
200
250
300
350
400
WAVELENGTH (mμ)

Table III - $R_f$ values of products produced by solar irradiation of psoralen under different conditions.

(Irradiation in all experiments was for six hours)

| Form in which psoralen was irradiated | Mean $R_f$ values of fluorescent products | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Psoralen solution in 1:1 alcohol+cysteine Hydrochloride(pH 7.0) | 0.023 (++) | - | - | 0.20 (+) | 0.33 (++) | - | 0.90 (+++) |
| Psoralen solution in 1:1 alcohol+cysteine Hydrochloride(pH 2-3) | 0.00 (++) | 0.036 (+) | 0.122 (+) | 0.17 (++) | 0.33 (+++) | 0.53 (++) | 0.90 (+++) |
| Psoralen suspension in dil.HCL, pH 2-3 | 0.017 (+) | - | - | 0.221 (++) | 0.405 (++) | 0.57 (+) | 0.90 (+++) |
| Psoralen suspension in Water | 0.008 (+++) | - | - | - | 0.37 (+) | - | 0.90 (+++) |
| Psoralen suspension in 0.2 M phosphate buffer pH 7.3 | 0.013 (+) | 0.071 (+) | - | - | - | - | 0.90 (+++) |
| Psoralen suspension in 0.2M phosphate buffer pH 8.3 | 0.011 (++) | - | - | 0.21 (+) | - | - | 0.90 (+++) |

*The intensity of fluorescent bands given in parenthesis is indicated as follows: +, faint; ++, medium; +++, strong.

between 300 and 420 mμ and showed a generalized absorption getting stronger towards shorter wavelengths. However, when cysteine at pH 7.0 was used three of the fluorescent compounds ($R_f$ 0.36, 0.1?? and 0.53) disappeared.

Irradiation of psoralen suspension in water, phosphate buffer (pH 7.3 and 8.3) and in dilute hydrochloric acid was also (Table III) indicated that the formation of fluorescent products under these conditions was very slow. When an aqueous suspension of psoralen was subjected to solar irradiation only one product ($R_f$ 0.37) was formed, besides that of psoralen, in addition of a very minor product having $R_f$ value of 0.008. With psoralen suspended in dilute hydrochloric acid, however, three fluorescent compounds of $R_f$ values 0.221, 0.405 and 0.570 were formed.

## DISCUSSION

The results of the present study would indicate that the irradiation of psoralen with ultraviolet or solar light resulted in the production of certain fluorescent compounds, other than psoralen. The amount of the products as judged by the intensity of the fluorescence was found to depend on the time of irradiation. Even half an hour solar irradiation produced a

number of products having different $R_f$ values. The intensity of these spots increased with the time of irradiation upto about 8 hours, after which no further increase was seen. These observations are similar to those of Fowlks who identified a dimer and furocoumaric acid as the irradiation products of psoralen. However, in our studies the absorption spectra of the eluates of different spots, had no resemblence with the absorption spectra of either the dimer or furocoumaric acid. Vessely and Minjaski[28] exposed 5,6 dimethoxyangelicin in a thin layer to diffused sunlight for 2.5 months and obtained a dimer which regenerated the parent compound upon being heated. In a study on photochemical reactions in sunlight Schonberg et al.[29] investigated the reaction of phenanthraquinone with a number of ethylene denvatives including 8-methoxypsoralen. He postulated the reaction as between 4',5' positions of the furocoumarin and the orthoquinone oxygens of phenanthaquinone to give a derivative of furobenzodioxin. The irradiation of 8-MOP has also been reported by Lerner et al. to bring about a loss of characteristic peaks of the absorption spectrum and to give rise to a generalized absorption which becomes stronger towards the shorter wavelength region. Our results are in close conformity to these findings. However, under the conditions used by us, all the psoralen probably did not get converted to the break down product and hence the irradiated

product, as a whole, still has the characteristic peaks exhibited by the parent compound. After chromatography, the eluate of the fluorescent products had the same absorption spectra (generalized absorption getting stronger towards the shorter wavelength region) as reported by Lerner for 8-MOP. The irradiation of 8-MOP has also been investigated in presence of flavin mononucleotide (FMN) using ultraviolet irradiation of maximum emission 3655$A^{0}$, and was found to give rise to spots of yellow colour and yellow fluorescence at low $R_f$ values (0.15-0.22) and spots with violet or blue violet fluorescence at $R_f$ values (0.85-0.90). The intensity of the new spots was seen to increase upto a maximum, with the time of irradiation. It has not been possible if the <u>in vitro</u> reaction between FMN and some furocoumarins could explain the mechanism of photodynamic activity on the human and guinea pig skin by furocoumarin. However the authors found strict parallelism between chemical reactivity and biological action, that is why the photodynamically active furocoumarin gave new compounds, when irradiated with solution of FMN as observed by paper chromatography. No new spot is detectable when photodynamically inactive derivatives were used.

Present studies also indicate that irradiation of psoralen with either solar or ultraviolet rays in presence of cysteine or other sulfhydryl compounds also

results in the production of fluorescent compounds other than psoralen. That fluorescent compounds ($F_f$ value of 0.37 with ultraviolet irradiation, and $F_f$ value of 0.39 with solar irradiation) having absorption spectrum similar to that obtained by irradiation of psoralen alone or with cysteine should be produced even after incubation of psoralen suspension with cysteine at pH 2-3 is rather interesting and would probably indicate the possible role of psoralen in the inactivation of sulfhydryl groups, which are important *in vivo* determinants of tyrosinase activity and hence melanin formation in the skin[30-32]. The mechanism by which 8-MOP may stimulate the suntanning process could possibly, be due to its accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing the melanin formation to proceed at a comparatively faster rate[33].

The formation of newer products from psoralen as a result of ultraviolet and solar irradiation also appears to be interesting in view of the fact that in the treatment of vitiligo, furocoumarins *per se* have been reported to be inactive unless aided by exposure to either ultraviolet or solar irradiation. As speculated earlier[19], it may be that one of the transformed products of psoralen may be responsible for accelerating melanin formation in vitiligo patients. This together with the photo-oxidation of inhibitors as sulphydryl groups of dopa to melanin, or a change in redox

potentials making the conditions more favourable for normal melanin formation in the presence of psoralen and ultraviolet or solar light, may be some of the mechanisms involved in the action of furocoumarins.

## SUMMARY

Irradiation of psoralen in suspension or aqueous ethanol solution with ultraviolet (maximum emission at 2537A°) and solar irradiation, with or without cysteine or glutathione has been shown to produce fluorescent products other than psoralen. In the case of ultraviolet irradiated psoralen the ultraviolet absorption spectrum of the major products ($R_f$ 0.62-0.67) has been found to be different from either furocoumaric acid or dimer of psoralen, but the product indicated a generalized absorption, getting stronger towards the shorter wavelength region. Incubation of psoralen with cysteine or reduced glutathione at acid pH likewise produced other fluorescent products.

In the case of solar irradiation, even 30 minutes gave rise to products having different $R_f$ values. The major products formed under the different conditions appears to be one giving bluish fluorescence ($R_f$ 0.36) and other having greenish bright fluorescence ($R_f$ 0.43). The intensity of these spots increased with the time of irradiation unto about 2 hours after which no further

increase was seen. The ultraviolet absorption spectra of these spots indicated a generalized absorption getting stronger towards the shorter wavelength region. Irradiation of psoralen in the presence of an aqueous solution of cysteine hydrochloride produces other fluorescent products. The possible significance of such transformation of psoralen in pigment production has been discussed.

REFERENCES


1. Edwards, E.A. and Duntley, S.Q., — Science, 92 (1939) 235.
2. Miescher, G., — Arch.Dermatol.Syph., 180 (1940) 238.
3. Blum, H.F. and Terus, W.S., — Am.J.Physiol., 144 (1945) 177.
4. Fitzpatrick, T.B., Becker, S.W.(Jr), Lerner, A.B. and Montgomery, H., — Science, 112 (1950) 223.
5. Lerner, A.B. and Fitzpatrick, T.B., — Physiol.Rev., 30 (1950) 91.
6. Rothman, S., Krysa, H.F. and Smiljanic, A.M., — Proc.Soc.Exp.Biol.Med., 62 (1946) 208.
7. Pathak, M.A., Riley, F.J., Fitzpatrick, T.B. and Curveir, W.L., — Nature, 133 (1962) 148.
8. Snell, R.S., — J.Invest.Dermatol., 40 (1963) 137.
9. Freeman, R.G., Cockerell, E.G., Armstrong, J. and Knox, J.M., — Ibid., 39 (1962) 295.

10. Knox, J.M., J.Chronic Diseases, 13 (1961) 391.

11. Snell, R.S., J.Invest.Dermatol., 40 (1963) 233.

12. Howell Clark, (Jr), Ibid., 37 (1961) 171.

13. Pathak, M.A., Ibid., 37 (1961) 397.

14. Musajo, L. and Rodighiero, G., Experientia, 13 (1962) 153.

15. Becker, S.W. (Jr), J.Invest.Dermatol., 32 (1959) 263.

16. Idem., Science, 127 (1958) 878.

17. Zimmerman, M.C., J.Invest.Dermatol., 32 (1959) 269.

18. Lerner, A.B., Denton, C.R. and Fitzpatrick, T.B., Ibid., 25 (1953) 299.

19. Fowlks, W.L., Ibid., 32 (1959) 249.

20. Stoll, V.A., Pereira, A. and Rentz. J., Helv.Chim.Acta, 33 (1950) 1637.

21. Musajo, L. and Rodighiero, G., Nature, 190 (1961) 1199.

22. Chakraborty, D.P. and Bose, P.K., J.Indian Chem.Soc., 33 (1956) 905.

23. Rodighiero, G., Caporale, G. and Ragazzi, E., Atti Ist.Veneto.Sci.Lettere Arti, 111 (1953) 195.

24. Swain, T., Biochem.J., 53 (1953) 200.

25. Riedl, K. and Neugebauer, L., Monatsh., 83 (1952) 1093.

26. Svendsen, A.B., Pharm.Acta Helv., 27 (1952) 44.

27. Nakabayashi, T., Tokoroyama, T., Miyazaki, H. and Isono, S., J.Pharm.Soc.Japan, 73 (1953) 669.

| | |
|---|---|
| 28. Wessely, G.Von and Dinjaski, K., | Monatsh., 64 (1934) 131. |
| 29. Schonberg, A., Latif, N., Moubasher, R. and Sina, R., | J.Chem.Soc., 1951 (1951) 1364. |
| 30. Lorincz, A.L., | Pigmentation in "The Physiology and Biochemistry of Skin", Ed. Rothman, S. The University of Chicago Press, Chicago, (1939), pp. 531-533. |
| 31. Hogeboom, G.H. and Adams, M.H., | J.Biol.Chem., 145 (1942) 273. |
| 32. Bernheim, F. and Bernheim, M.L.C., | Ibid., 145 (1942) 213. |
| 33. Joseph Juttis, | J.Am.Pharm.Assoc., 49 (1960) 447. |

# CHAPTER III

## INACTIVATION OF SULFHYDRYL GROUPS BY IRRADIATED PSORALEN

Many organic sulfur compounds, containing reactive sulfhydryl groups, have been shown to be strong inhibitors of tyrosine-tyrosinase reaction, and hence melanin formation. Among them are diethyldithiocarbamate, cysteine, glutathione, 2,3-dithiopropanol (BAL), thiourea and its derivatives, e.g. as phenylthiourea, allylthiourea, $\alpha$-napthylthiourea and thiouracil[1-5]. Thiourea and its derivatives have been reported to be not only active inhibitors of melanin formation *in vivo*, but also have been shown to be active *in vitro* in lower vertebrates[6] and cultured cells of chick melanocytes[7]. Among mammals, black rats, fed with $\alpha$-napthylthiourea or phenylthiocarbamide have been observed to indicate greying of hair, only as long as such substances remain in the diet[8,9]. In human beings too, these compounds usually lead to inhibited pigment formation. Thus thiouracil, given to a patient with metastatic malignant melanoma, stopped the associated melanuria[10]. Again a negro patient treated with thiouracil for hyperthyroidism was reported to develop areas of depigmentation[11].

It has been suggested that degree of melanin pigmentation in organisms may perhaps be regulated by inhibitory sulfhydryl compounds. It was shown that crude aqueous extracts of rabbit or guinea pig skin could inhibit the oxidation of tyrosine by tyrosinase[12-14]. Rothman[15] and coworkers were able to demonstrate this inhibitory factor in human epidermis and further showed that its inhibitory action could be reversed by specific sulfhydryl poisons. The inhibition of melanin formation with skin extracts was found to be proportional to the logarithm of the molar concentration of sulfhydryl[16]. Rothman concluded that the inhibition was a function of sulfhydryl compounds present in the epidermis and proposed the following hypothesis to account for the natural regulation of melanin formation. In melanocytes, although both substrate (tyrosine) and active enzyme are present, they are unable to interact fully because of the inhibitory action of the -SH groups present. Pigmentogenic stimulii, such as sunshine, X-rays, heat and inflammatory skin diseases, act by oxidizing or otherwise destroying the inhibitory sulfhydryl groups and thus permitting the enzyme to act freely on substrate.

Furocoumarins, along with solar and ultraviolet irradiation has been used in the treatment of leucoderma.

However their exact mechanism of action is not yet clear. It was reported that methoxsalen (8-methoxy-psoralen) as such or under irradiation had no effect on tyrosine-tyrosinase reaction[17], although Chakraborty et al.[18] observed *in vivo* enhancement of tyrosinase action and melanin formation in the skin and liver tissues of male toads after oral administration of psoralen. Pathak and Fowlks[19] have shown that application of 100 mg of psoralen and methoxsalen on the back of guinea pigs, being followed by exposure to ultraviolet light or direct sunlight resulted in a marked decrease in succinic dehydrogenase activity, which was not as great as that observed after oral administration of the drug. Further no conclusive decrease of sulfhydryl group containing compounds e.g. cysteine and reduced glutathione[20] was observed after irradiation in presence of furocoumarin. Coumarins *per se* are known to inhibit sulfhydryl groups, and the action of certain aromatic thiols on coumarins has also been reported[21]. The possibility of inactivation of sulfhydryl group by furocoumarins and radiant energy probably need special attention, in order to understand its mechanisms of action. It was therefore considered of interest to investigate the inactivation, if any, of sulfhydryl groups by normal and irradiated

psoralen. The results of such studies are reported in the present chapter.

The inactivation of succinic oxidase, an -SH containing enzyme[22], was taken as the index of inactivation of sulfhydryl groups. Succinic dehydrogenase, present in the epidermis of all mammals[23-26] has been shown to be inhibited by those substance which are known to inactivate the sulfhydryl groups[27,28]. It was shown by Hopkins and Morgan[22] that activity of succinic dehydrogenase is completely abolished when its preparation is incubated in a solution of oxidized glutathione (GSSG) at pH 7.6. When the inactive enzyme was reincubated with reduced glutathione (GSH), the activity was found to be completely restored. Alloxan in low concentrations, as an oxidant of -SH groups, has also been seen to inactivate the succinic oxidase. Maleic acid and iodoacetic acid, capable of reacting with -SH groups, also have been found to inactivate the enzyme[29]. Further sulfhydryl combining compounds including oxidizing agents, o-iodosobenzoate, oxidized glutathione, p-aminophenylarsenoxide and p-chloromercuribenzoate have been shown by Slater[30] to inactivate the succinic oxidase system. A large number of heavy metals have also been found to inhibit succinoxidase and the inhibition has been explained on the basis of

combination of metals with the -SH groups of the protein moiety[31]. Further proof therefore for the inactivation of -SH groups was sought by inhibiting the potato tyrosinase with thiourea and then its reversal by normal and irradiated psoralen and these results have also been included in the present chapter.

## METHODS AND MATERIALS

### Concentration of psoralen

A solution of psoralen (1 mg/ml) in 1:1 alcohol was prepared and 10 ml was irradiated under bright sun in 50 ml stoppered cylinder, as described in Chapter II. After irradiation the solution was transferred to a 9.0 cm glass petri dish which was then placed under infra-red lamp (Philips Infraphil, Type 13373F/479, 230V, 150W) for concentration.

### Succinic oxidase

Rat kidney was taken as the source of the enzyme. Healthy rats weighing about 100 gm from Central Drug Research Institute Colony were selected, killed by a blow on the head. Kidneys were immediately removed, and after removal of the adherent tissue, were chilled in a beaker dipped in ice bath. The homogenate was

then prepared (10% weight/volume) in ice cold M/15 phosphate buffer pH 7.0, by means of Potter Elvehjem homogenizer, which was previously cooled in freezing mixture.

2.0 ml of 10% homogenate was added to 2.0 ml of irradiated psoralen (6.0 mg) solution. 1.0 ml of the mixture was added to each flask. A control without psoralen was also carried out. Manometric methods according to Slater[32] were used for the assay of the enzyme. A uniform shaking rate of 100 strokes per minute and a constant temperature of 37 ± 0.1°C were maintained throughout the experiment. All reagents were adjusted to pH 7.0 before use. Each flask contained 1.0 ml M/5 phosphate buffer (pH 7.0), and 1.0 ml kidney homogenate in the main compartment, 1.0 ml of M/10 succinic acid (pH 7.0) in side arm and 0.2 ml of 10 per cent potassium hydroxide solution in the centre cup with a 2.0 sq.cm filter paper. The flasks were equilibrated for 10 minutes, and subsequently the substrate was tipped into the main compartment. Readings were taken at intervals of 15 minutes.

Tyrosinase.

To the 6.0 mg irradiated psoralen (concentrated to 1.0 ml) were added 1.0 ml of thiourea (10 µg/ml)

and both were incubated for two hours at 37°C in an incubator. Proper controls were simultaneously included.

Fresh potatoes were washed with water and were cut into small pieces by means of a sharp knife. They were kept at -13°C for 3 to 4 hours, ground in a previously chilled pestle and mortar and the juice was obtained by squeezing through a fine cloth. 2 ml of potato juice was added to each of the tubes containing irradiated psoralen and thiourea, thiourea and water.

Tyrosinase was again estimated by conventional manometric technique. 1 ml of the potato juice and 1 ml of M/5 phosphate buffer (pH 6.8) was placed in the main compartment of the Warburg flask, 1.0 ml of pure L-tyrosine in M/5 phosphate buffer (pH 6.8) was added to the side arm. The inner cup contained 0.2 ml of 10% Potassium hydroxide solution and a filter paper (2.0 sq.cm Whatman No.1) for absorbing any carbon dioxide evolved during oxidation. After equilibration for 10 minutes the substrate was tipped into the main compartment and the oxygen uptake was determined at 30 minutes interval. Average readings from two flasks were calculated in each experiment. Control flasks, without tyrosine were always included in each experiment and the nett oxygen consumption was calculated after substraction of the endor-nous value.

## RESULTS

Table I represents the succinic oxidase activity in presence of psoralen either in solution or as suspension and psoralen irradiated for 30 minutes and slight increase in the oxidation of succinate is observed in all cases. The average increase with solution, suspension and 30 minutes irradiated psoralen were 3.9, 14.11 and 6.64 per cent respectively.

Table II represents the oxidation of succinate in presence of psoralen irradiated for the periods ranging from one to six hours. The microlitres of oxygen consumed at all the periods was found to be significantly decreased when compared with control in the presence of psoralen irradiated for one, two, three, four and six hours. The inhibition of succinic oxidase brought about by irradiated products of psoralen was found to increase with the period of irradiation. The average inhibition (Table III) after one hour exposure was 14% which increased to 55% after psoralen was irradiated for two hours. Inhibition amounting to 61%, 69% and 70% respectively was obtained when three, four and six hours irradiated psoralen was added to the reaction mixture.

Table I - Effect of psoralen on the metabolism of succinate by rat kidney homogenate.

| Time (minutes) | µl. of oxygen consumed | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | Psoralen suspension in water | | Psoralen supernatant in water | | Psoralen irradiated for half an hour | |
| | Endo. | Succ. | Endo. | Succ. | Endo. | Succ. | Endo. | Succ. |
| 15 | 15.47 | 228.78 | 15.34 | 368.51 (14.00) | 17.70 | 239.04 (3.73) | 21.33 | 236.43 (0.94) |
| 30 | 32.13 | 427.80 | 18.88 | 478.65 (16.20) | 28.72 | 439.19 (3.74) | 15.19 | 429.51 (5.22) |
| 45 | 36.89 | 595.20 | 33.04 | 679.56 (15.80) | 38.94 | 619.08 (3.91) | 7.58 | 599.61 (6.04) |
| 60 | 48.79 | 719.82 | 35.40 | 798.43 (13.71) | 46.02 | 735.38 (2.73) | 16.93 | 737.49 (6.64) |
| 75 | 54.74 | 829.56 | 36.58 | 907.10 (13.82) | 49.56 | 849.92 (3.29) | - | 835.02 (7.77) |
| 90 | 59.50 | 914.26 | 44.84 | 994.90 (11.15) | 51.92 | 957.82 (5.98) | - | 919.46 (7.57) |

The values in parenthesis represent the per cent increase.

Endo. = Endogenous; Succ. = Succinate.

Table II - Effect of irradiated psoralen on the metabolism of succinate by rat kidney homogenate.

| Time (minutes) | µl. of oxygen consumed. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | | A | | B | | C | | D | | E | |
| | Endo. | Succ. | Endo. | Succ. | Endo. | Succ. | Endo. | Succ. | Endo. | Succ. | Endo. | Succ. |
| 15 | 3.72 | 114.54 | 4.87 | 96.27 (17.53) | - | 50.82 (54.14) | 1.21 | 42.90 (61.48) | 1.18 | 37.30 (67.41) | 1.24 | 36.64 (68.70) |
| 30 | 6.20 | 204.18 | 8.67 | 186.62 (15.17) | - | 88.90 (55.10) | 2.28 | 77.48 (62.02) | 1.18 | 63.50 (68.53) | - | 62.00 (69.68) |
| 45 | 10.56 | 287.18 | 5.37 | 240.09 (14.43) | 1.47 | 130.58 (54.78) | 2.36 | 109.11 (61.41) | 2.36 | 189.40 (68.54) | 1.24 | 84.74 (69.04) |
| 60 | 13.64 | 346.94 | 4.29 | 288.46 (14.74) | 2.82 | 143.94 (57.66) | 1.16 | 127.45 (62.11) | 1.18 | 110.70 (67.14) | 2.48 | 94.48 (72.09) |
| 75 | 16.12 | 390.60 | 4.09 | 330.97 (12.71) | 2.68 | 164.72 (56.73) | 4.34 | 150.20 (61.05) | - | 124.90 (66.66) | 2.48 | 115.49 (69.82) |
| 90 | 19.84 | 458.16 | 3.38 | 392.70 (11.17) | 4.34 | 194.14 (56.70) | 6.84 | 180.86 (60.30) | 2.36 | 135.70 (69.58) | 1.24 | 132.04 (17.18) |

The data in the columns A,B,C,D and E represent the microlitres of oxygen consumed with one, two, three, four and six hours irradiated psoralen respectively.
The values in the parenthesis represent the per cent inhibition.

Table III - Percentage inhibition of succinic oxidase brought about by psoralen irradiated for different periods.

| Irradiation time (hours) | Percentage inhibition (average) |
|---|---|
| 1 | 14.39 |
| 2 | 55.67 |
| 3 | 61.39 |
| 4 | 67.97 |
| 6 | 69.75 |

From the graphical representation of the data (Fig.1) it is clearly seen that the inhibition of succinic oxidase brought about by irradiated products of psoralen increases with the period of irradiation. The changes caused by irradiation in psoralen seem to be complete after 4 hours exposure, since the inhibition caused by four and six hours irradiated psoralen were observed to be almost of the same order.

Since the irradiated and concentrated solution of psoralen, in these studies, was in the state of fine suspension, it was of interest to investigate

Fig. 1 Inhibition of succinic acid oxidation by irradiated psoralen.

A - Control

B - 1 hour irradiation

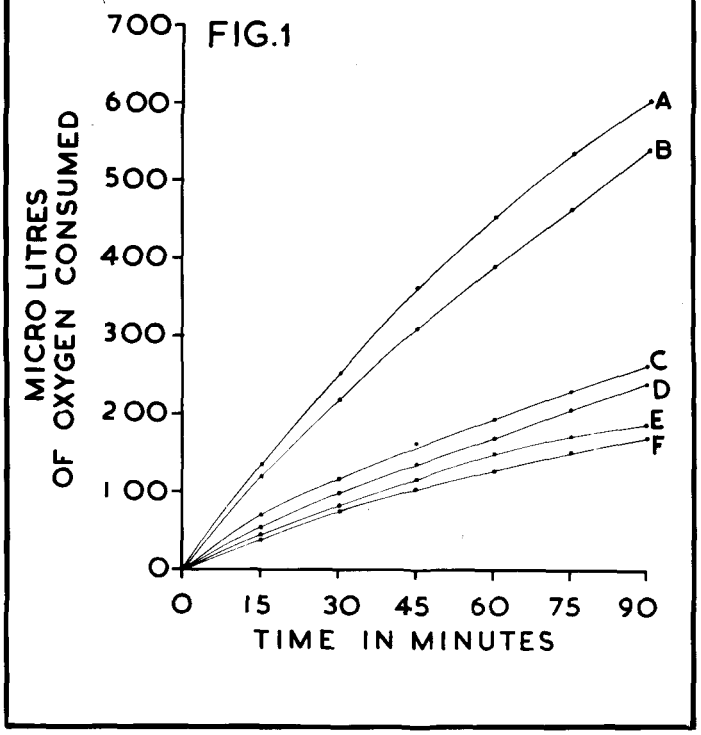
C - 2 hours irradiation

D - 3 hours irradiation

E - 4 hours irradiation

F - 6 hours irradiation

FIG.1


700
600
500
400
300
200
100
0
MICRO LITRES
OF OXYGEN CONSUMED
A
B
C
D
E
F
0
15
30
45
60
75
90
TIME IN MINUTES

the inhibition caused by soluble and sedimented portions separately. 9.0 ml aliquot of concentrated psoralen, was divided in three portions of 3.0 ml each. One was kept as such while the other two were centrifuged at 3,000 rpm for 20 minutes in an International Refrigerated Centrifuge (Model HR-1) using Rotor 856. After centrifugation, the contents of one of the two was again mixed while the supernatant of the other was separated, the residue was suspended in 3.0 ml of distilled water. Two ml of each solution was then added to 2.0 ml of kidney homogenate and 1.0 ml of the mixture was added to each Warburg flask and the enzyme activity was as usual followed.

The data obtained (Table IV) shows that psoralen irradiated for six hours brought about an inhibition of 64 per cent as usual, centrifugation and then resuspension did not bring about any change. However supernatant share was seen to about one third of the inhibition and the rest was shared by the residue. In the latter period of succinate oxidation the per-centage inhibition was seen to decrease slightly.

Table IV - Effect of irradiated psoralen on the metabolism of succinate by rat kidney homogenate.

| Time (minutes) | µl of oxygen consumed. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | | Irr. Psoralen | | Resuspended | | Supernatant | | Residue | |
| | Endo. | Succi. | Endo. | Succi. | Endo. | Succi. | Endo. | Succi. | Endo. | Succi. |
| 15 | 9.52 | 236.22 | 9.92 | 90.75 (64.72) | 6.78 | 85.65 (65.66) | 11.20 | 204.49 (15.86) | 14.24 | 129.98 (49.62) |
| 30 | 15.47 | 422.22 | 16.12 | 163.35 (63.81) | 19.21 | 152.88 (67.14) | 30.40 | 337.51 (12.22) | 30.26 | 246.68 (46.30) |
| 45 | 26.18 | 571.02 | 24.80 | 234.30 (61.74) | 30.51 | 210.00 (67.06) | 43.20 | 506.22 (15.02) | 39.16 | 327.96 (47.02) |
| 60 | 33.32 | 675.18 | 21.08 | 264.00 (62.14) | 20.34 | 250.32 (64.17) | 32.00 | 612.04 ( 9.64) | 32.04 | 388.00 (44.55) |
| 75 | 38.08 | 757.02 | 29.76 | 320.10 (59.62) | 29.33 | 302.40 (62.03) | 44.90 | 706.42 ( 7.98) | 44.50 | 457.84 (42.51) |
| 90 | 44.03 | 837.00 | 47.08 | 363.00 (60.16) | 35.03 | 346.08 (60.78) | 51.20 | 772.00 ( 9.11) | 46.28 | 509.28 (41.74) |

The values in the parenthesis represent the per cent inhibition.

Endo. = Endogenous; Succi. = Succinate.

## Reversal of thiourea inhibition of potato tyrosinase by irradiated psoralen

Further evidence for the inactivation of sulfhydryl groups by the products of irradiated psoralen was attempted by incubating thiourea (10 µg) with psoralen irradiated for different periods and seeing its effect on potato tyrosinase. Table V represents the oxidation of tyrosine in presence of thiourea, thiourea and psoralen suspension. The microlitres of oxygen consumed after two hours of incubation was 298 in control and 189 and 194 with thiourea and thiourea plus psoralen, indicating thereby that psoralen suspension *per se* is not significantly effective in bringing about the reversal of enzyme activity, inhibited by thiourea.

Table V - Reversal of thiourea inhibition of potato tyrosinase by psoralen suspension.

| Time (min.) | µl. of oxygen consumed. | | | | | |
|---|---|---|---|---|---|---|
| | Control | | Thiourea | | Thiourea+Pso. | |
| | Endo. | Tyro. | Endo. | Tyro. | Endo. | Tyro. |
| 30 | 4.96 | 77.40 | 9.00 | 40.10 (57.08) | 10.01 | 41.50 (56.53) |
| 60 | 9.92 | 165.50 | 12.60 | 91.66 (49.19) | 15.73 | 91.95 (50.94) |
| 90 | 13.64 | 239.70 | 12.60 | 140.92 (43.24) | 12.87 | 137.19 (45.01) |
| 120 | 18.60 | 298.30 | 16.20 | 189.84 (36.28) | 15.73 | 194.34 (39.72) |

The values in parenthesis represent the per cent inhibition.

Table VI represents the microlitres of oxygen consumed in presence of thiourea plus psoralen irradiated for half, one, three and six hours. It is evident from the data that irradiated psoralen could bring about the reversal of the thiourea inhibition, the extent of which depends on the period of irradiation. The percentage inhibition of tyrosine oxidation in presence of thiourea and thiourea plus psoralen irradiated for different periods have been given in TableVII, from which it is clear that thiourea, (in concentration used) brought about an inhibition of 56.1 per cent in the tyrosinase activity (as seen by reduced oxygen uptake) in the initial period of oxidation. However when thiourea incubated with irradiated psoralen was added, the inhibition was respectively reduced to 43.6%, 34.52%, 27.52% and 21.71% with half, one, three and six hours irradiation of psoralen. The results also show that the inhibition by thiourea decreased with time of incubation (from 56.1% in 30 minutes to 38.00% in 120 minutes) and the same pattern was obtained with thiourea and psoralen irradiated for different periods (30.6%, 18.3%, 12.65% and 7.92% by psoralen irradiated for half, one, three and six hours exposure respectively in 120 minutes).

Table VI - Reversal of thiourea inhibition of potato tyrosinase by psoralen irradiated for different periods.

| Time (minutes) | µl. of oxygen consumed. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | | Thiourea | | A | | B | | C | | D | |
| | Endo. | Tyro. | Endo. | Tyro. | Endo. | Tyro. | Endo. | Tyro. | Endo. | Tyro. | Endo. | Tyro. |
| 30 | 7.44 | 96.10 | 12.98 | 55.45 | 10.01 | 54.69 | 9.22 | 66.27 | 8.05 | 72.31 | 9.25 | 78.66 |
| 60 | 13.64 | 185.00 | 21.24 | 108.46 | 17.16 | 115.60 | 12.95 | 138.92 | 13.35 | 147.85 | 12.79 | 157.92 |
| 90 | 22.32 | 251.00 | 25.96 | 144.49 | 18.59 | 157.78 | 19.72 | 198.17 | 20.33 | 206.06 | 21.75 | 229.62 |
| 120 | 37.20 | 306.20 | 43.66 | 210.43 | 30.03 | 211.30 | 31.76 | 251.54 | 30.45 | 265.41 | 35.23 | 282.92 |

The data in the columns A,B,C and D represent the microlitres of oxygen consumed with half, one, three and six hours irradiated psoralen plus thiourea respectively.

Endo. = Endogenous; Tyro. = Tyrosine.

Table VII - Percentage inhibition of tyrosine oxidation in presence of thiourea, and thiourea plus psoralen irradiated for different periods.

| Time (minutes) | Percentage inhibition | | | | |
|---|---|---|---|---|---|
| | Thio-urea | A | B | C | D |
| 30 | 56.10 | 49.60 | 34.52 | 27.52 | 21.71 |
| 60 | 49.10 | 46.13 | 26.49 | 21.51 | 15.31 |
| 90 | 43.79 | 41.32 | 21.97 | 18.74 | 9.10 |
| 120 | 38.00 | 32.61 | 19.30 | 12.65 | 7.92 |

The data in the columns A,B,C and D represent the per cent inhibition with half, one, three and six hours irradiated psoralen plus thiourea respectively.

With a view to have a better comprehension, the percentage activity with respect to the control has been presented graphically in Fig.2, which would show that the reversal is greater in the later stages than at the beginning. This may be because, the estimation of tyrosinase activity in presence of thiourea and irradiated products of psoralen, in itself, involves further incubation of these materials together.

Fig. 2 Reversal of thiourea inhibition of tyrosinase by psoralen irradiated for different periods.

A, B, C, and D represent activity at 30, 60, 90 and 120 minutes respectively.

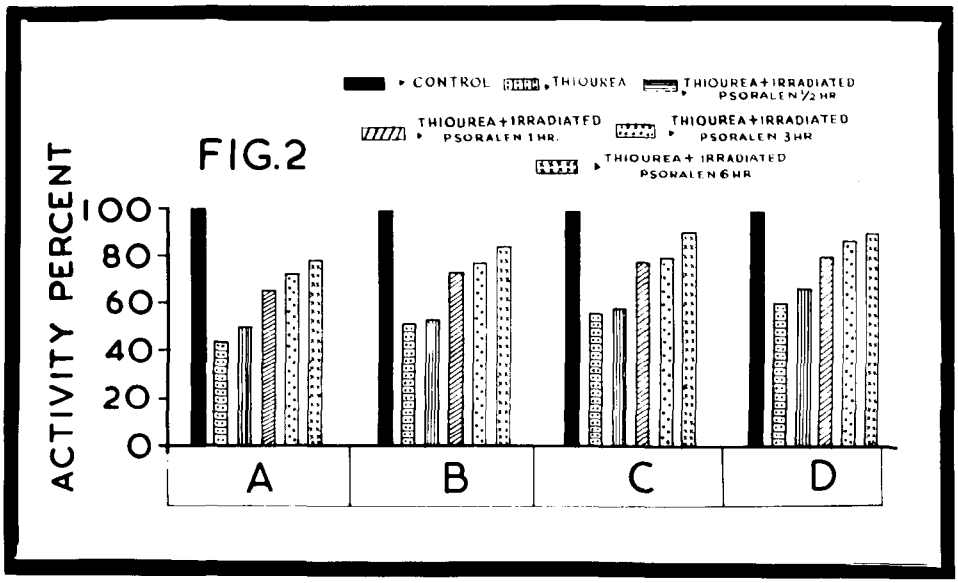

CONTROL
THIOUREA
THIOUREA + IRRADIATED PSORALEN ½ HR
THIOUREA + IRRADIATED PSORALEN 1 HR.
THIOUREA + IRRADIATED PSORALEN 3 HR
THIOUREA + IRRADIATED PSORALEN 6 HR
ACTIVITY PERCENT
100
80
60
40
20
0
A
B
C
D

FIG.2

## DISCUSSION

Inspite of the fact that sulfhydryl groups have been considered important *in vivo* determinants of tyrosinase activity, little information is available regarding their exact mechanism *vis-a-vis* the role of ultraviolet light and furocoumarins in pigment production. It has been shown that oxidation of tyrosine or dopa by polyphenol oxidase in the presence of excess reduced glutathione or cysteine leads to the formation of an addition compound of dopa quinone with the thiol[33]. Mofty et al.[34] has shown that following administration of 8-methoxypsoralen and 8-Isoamyleneoxypsoralen in doses ranging from 200, 400 and 800 mg/kg of body weight, there were no variation in blood sugar and glutathione. Pathak and Fowlks[19] applied 100 µg of psoralen or 8-MOP on the skin of guinea pigs, followed by exposure to ultraviolet light or direct sunlight, which resulted in a marked decrease in succinic dehydrogenase activity.

Rothman[35] has pointed out that the effect of ultraviolet irradiation was chiefly the inactivation of inhibitors, like -SH compounds, in skin leading to increased tyrosinase activity and better pigmentation. Following ultraviolet irradiation of the skin, the

level of inhibitory sulfhydryl groups (which form strong covalent bond with copper) was also found to be reduced[15]. Further it was shown[36] that hyperpigmented skin in post inflamatory conditions has about half the sulfhydryl content of the sorrounding skin and that vitiliginous skin has about twice as high a sulfhydryl content. The author, giving two interpretations has indicated that either the high sulfhydryl content prevents melanin formation by inhibiting tyrosinase activity or sulfhydryl may accumulate secondarily because no melanin is being formed, a process which would use up sulfhydryl group. Many of the intermediates in the melanin forming chain of reactions are known to react with such groups.

The results of the present study would show that irradiated psoralen has the capacity to inhibit succinic oxidase, an -SH containing enzyme. That this is caused by the action of some of these products on sulfhydryl groups of the enzyme is indicated by the abolition of thiourea inhibition of tyrosinase by irradiated psoralen. Since tyrosinase is known to be involved in melanin formation it seems plausible that the pigmentation by psoralen may depend on the interaction of psoralen or more correctly its irradiation product or products with sulfhydryl groups inhibitory to tyrosinase. This would explain the role of ultraviolet or solar radiations as potentiators of psoralen effect. The inability of psoralen per se to bring

about pigment production and the observations of Lerner et al.[37] that methoxsalen as such has no effect on tyrosinase are in conformity with the present observations.

In this connection it might also be interesting to mention that some trace metals, when present in excessive amounts in the skin, are often associated with increased melanin formation. These include iron, silver, gold, bismuth and arsenic[38]. It has been postulated that the metals cause melanosis by binding tissue sulfhydryl groups, which normally keep tyrosinase in a partially inhibited state. Deficiencies of Vitamin A[39], C[40] and niacin[41] have all been reported to lead increased melanin formation. The melanosis in vitamin A deficiency occurs at the sites of the follicular hyperkeratotic papules. It has been suggested that the local reduction in sulfhydryl groups accompanying increased keratinization may serve to release at these points the normal sulfhydryl inhibition of tyrosinase. The melanosis occasionally associated with scurvy can also probably be accounted for on the basis of release of sulfhydryl inhibition. Melanosis by ACTH, hypothyroidism, and adrenal cortex has also been explained on the basis of sulfhydryl tyrosinase inter-relationship[1].

## SUMMARY

The inactivation of -SH groups has been studied with normal and irradiated psoralen. Since succinoxidase is an -SH containing enzyme, its inactivation was taken as an index for the inactivation of -SH groups. It was observed that psoralen either in solution or as suspension and psoralen irradiated for 30 minutes brought about a slight activation of the oxidation of succinate by rat kidney homogenate. Psoralen irradiated for one, two, three, four and six hours has found to bring about an inhibition of succinoxidase, the extent of which was seen to depend upon the period of irradiation of psoralen. Since psoralen solution after irradiation and concentration was in the form of fine suspension, the behaviour of supernatant and residue was also studied. Supernatant shared about one third of the inactivation and the remaining inhibition was given by the residue.

Further evidence of the inactivation of -SH groups by the products of irradiated psoralen was attempted by incubating thiourea (10 µg) with psoralen irradiated for different periods and observing its effect on potato tyrosinase. Psoralen suspension per se was not able to bring about the reversal of enzyme activity inhibited by thiourea. Irradiated psoralen,

however, could significantly bring about the reversal of thiourea inhibition, the extent of such reversal depended on the period of irradiation of psoralen. With six hours irradiated psoralen, almost complete reversal was obtained, after two hours of incubation.

REFERENCES


1. Lerner, A.B. and Fitzpatrick, T.B., Physiol.Rev., 30 (1950) 91.
2. Hogeboom, G.H. and Adams, M.H., J.Biol.Chem., 145 (1942) 273.
3. Bernheim, F. and Bernheim, M.L.C., ibid., 145 (1942) 213.
4. DuBois, K.P. and Erway, W.F., ibid., 165 (1946) 711.
5. Paschkis, K.E., Cantarow, A., Hart, W.M. and Rakoff, A.E., Proc.Soc.Exp.Biol.Med., 57 (1944) 37.
6. Jaques, R., Experientia, 6 (1950) 148.
7. Markert, C.L., Physiol.Zool., 21 (1948) 309.
8. Richter, C.P. and Clisby, K.H., Proc.Soc.Exp.Biol.Med., 48 (1941) 694.
9. Dieke, S.H., Endocrinology, 40 (1947) 123.
10. White, A.G., J.Lab.Clin.Med., 32 (1947) 1254.

11. Hellerstein, H.K., — Personal communication, cited by Lerner and Fitzpatrick (1).

12. Schaaf, F., — Arch.Dermatol.Syph., 175 (1938) 646.

13. Schaumann, K. and Danneel, R., — Biol.Zentr., 58 (1938) 949.

14. Ginsberg, B., — Genetics, 29 (1944) 176.

15. Rothman, S., Krysa, H.F. and — Proc.Soc.Exp.Biol.Med., 62 (1946) 208.

16. Flesch, P., — ibid., 70 (1949) 136.

17. Korting, G.W., Friederich, H.C. and Adams, W., — Dermatol.Wochenschr., 38 (1958) 934.

18. Chakraborty, D.P., Deb, C. and Mukherjee, M., — Sci.Cult.(Calcutta), 25 (1959) 396.

19. Pathak, M.A. and Fowlks, W.L., — J.Invest.Dermatol., 37 (1961) 183.

20. Musajo, L. and Rodighiero, G., — Experientia, 18 (1962) 153.

21. Mustafa, A., Kamel, M. and Allam, M.A. et al., — J.Am.Chem.Soc., 78 (1956) 5011.

22. Hopkins, F.G. and Morgan, E.J., — Biochem.J., 32 (1938) 611.

23. Serri, F., — Boll.Soc.Med.Chir.Pavia, 3-4 (1955) 1.

24. Formisano, V.P. and Montagna, W., — Anat.Record, 120 (1954) 993.

25. Braun-Falco, O. and Rathjens, B., — Arch.Klin.Exp.Dermatol., 201 (1955) 73.

26. Steigleder, G.K., — ibid., 207 (1958) 209.

27. Bonner, W.D.(Jr), — Biochem.J., 56 (1954) 274.

28. Stoppani, A.O.M. and Brignone, J.A., — Biochem.J., 84 (1956) 196.

29. Hopkins, F.G., Morgan, E.J. and Lutwak-Mann, C., — Ibid., 32 (1938) 1829.

30. Slater, E.C., — Ibid., 45 (1949) 130.

31. Barron, E.S.G., — 'Thiol groups of biological importance'. In Advances in Enzymology, Vol.11., Interscience Publishers Inc., New York (1951).

32. Slater, E.C., — Biochem.J., 45 (1949) 1.

33. Simmone Bouchilloux and Annie Mayrargue, — Bull.Soc.Chim.Biol., 42 (1960) 1045.

34. El-Mofty, A., El-Mofty, A.M., Abdelal, H. and El-Hawary, M.F.S., — A.M.A. Arch.Dermatol., 80 (1959) 53.

35. Rothman, S., — 'Physiology and Biochemistry of skin', Chicago, The university of Chicago Press, 1954.

36. Van Scott, E.J., Rothman, S. and Greene, C.R., — J.Invest.Dermatol., 20 (1953) 111.

37. Lerner, A.B., Denton, C.R. and Fitzpatrick, T.B., — Ibid., 20 (1953) 299.

38. Ormsby, O.S. and Montgomery, H., — 'Diseases of the skin' Philadelphia: Lea & Febiger, 1937.

39. Mu, J.W., Frazier, C.N. and Pillat, A., — Chinese J. Physiol., 11 (1937) 347.

40. Cornbleet, T., — Arch.Dermatol.Syph., 35 (1937) 471.

41. Harris, S. and Harris, S. (Jr), — 'Clinical Pellagra' St. Louis: C.V.Mosby Co. (1941).

# CHAPTER IV

## PHOTO-OXIDATION OF DIHYDROXYPHENYLALANINE (DOPA) IN PRESENCE OF DEOPALEN

Study of melanin formation may be said to have been started after Bloch's discovery of the famous dopa reaction[1-3], wherein he immersed frozen sections of pigmented human skin in a dilute solution of L-3, 4-dihydroxyphenylalanine at pH 7.3-7.4 and observed that brown granules appeared in the cytoplasm of the scattered cells in the basal layer. Bloch further correlated the intensity of this reaction with melanin forming capacities of skin from different sources, and concluded melanin to be a polymerized enzymatic oxidation product of dopa, mediated by the hypothetical pigmentogenic enzyme, dopa oxidase of the skin. This dopa oxidase concept, however was challenged[4] by a group of workers who pointed out that cells, other than melanin forming cells, were dopa positive including leukocytes, nerve fibrils, sweat glands, red blood corpuscles etc. This reaction was seen to proceed not only with dopa but also given by other compounds including p-hydroxyphenylpyruvic acid, 3,4-dihydroxyphenylpyruvic acid[5], adrenaline, p-cresol and tyrosine[6].

The biochemistry of melanin formation (cf. General Introduction) is now well established. Starting from tyrosine[7-10] the reaction consists in the oxidation of tyrosine to dopa by tyrosinase; oxidation of dopa to dopa quinone; its condensation to a leuco compound; and oxidation to dopachrome, decarboxylation and reduction to 5,6-dihydroxyindole; oxidation to indole 5,6-quinone and finally polymerization and oxidation to dopa melanin. In vivo, the quinones are known to react readily, before or after polymerization, with -SH and-$NH_2$ groups of protein to form a melanoprotein compound.

The formation of melanin has been known to be influenced by a number of factors such as copper, an essential factor for tyrosinase action[11-13], sulfhydryl groups, which inhibit and thus regulate the tyrosine-tyrosinase reaction[14,15], various hormones[16-18] such as MSH, thyroid, adrenal and gonadal; temperature, pH[14] and oxidation reduction potential[19].

Certain juices and extractives of plant such as parsley, celery, figs and parsnip[21,21] after contact with the skin and exposure to sunlight have been shown to cause areas of erythema and hyperpigmentation. All these extracts have now known to contain furocoumarins which are considered as the active agents. This activity of the furocoumarins coupled with their utility in the treatment of leucoderma stimulated considerable

interest among the research workers in different parts of the world. Particularly active in this field have been Musajo and Coworkers in Italy[22-24], the American group at the University of Oregon, namely Lerner, Pathak, and Fitzpatrick[25-26], and the Egyptian group at the University of Cairo[27-29]. The American group has been mainly interested in the more fundamental aspects of the photodynamic action, such as biochemistry of melanin formation[7,14] the mechanism of photodynamic effect[30], the inhibition of enzyme systems[31], and sensitization of bacteria[32,33] by furocoumarins. The Italian group has of late been trying to determine the mechanism of action of these compounds[34]. The Egyptian group isolated the active furocoumarins and have given more emphasis to the clinical aspect of the drug.

With regard to the photosensitizing properties, the parent compound psoralen, as pointed out by Musajo and Rodighiero[20], has the maximum activity and that the various structurally related compounds namely Bergapten, Isobergapten, Angelicin etc. are less active, depending on the ring system and nature of substituents. According to Fowlks[27], the primary event of photosensitization is the absorption of light by the photosensitizer with protein or nucleic acid. Pathak and Fellman[35] have, in addition, reported that all biologically active

furocoumarins, inducing photosensitization, possess absorption and fluorescence peaks at 320-360 and 420-460 mμ respectively. Other furocoumarins having fluorescent peaks outside this range have mostly been found to be inactive.

It is significant to mention here that furocoumarins have definitely been shown not to act by a photooxidative mechanism, although other photosensitizing molecules such as hematoporphyrins are known to exert by way of this mechanism. Musajo et al.[36] studied the photo-oxidation of α-terpinene to ascaridol, in presence of certain photodynamic compounds including furocoumarins. The irradiation was done under both visible and ultraviolet light. The photosensitizing property of furocoumarins when compared with other compounds was found to be insignificant. The same workers also studied the photodynamic haemolysis of red cells[37] and photo-oxidation of blood serum proteins[38] in presence of furocoumarins and some other well known photosensitizers such as hematoporphyrins, chlorophyll, methylene blue, Bengal rose, eosin etc. These studies again showed that furocoumarins, in contrast to other substances, have little or no photo-oxidizing action. This diversity[20] was explained on the basis that furocoumarins had photodynamic properties in a way, which was different from that of the

other groups, e.g. those compound which can photo-oxidise terpinene, cause haemolysis, photo-oxidize blood serum proteins are essentially not active if painted on the skin. On intradermical injection however, they cause an immediate, though short, photoreaction. Furocoumarins, on the other hand, do not influence the photo-oxidation of terpinen, do not cause haemolysis, do not photo-oxidize blood serum proteins, but on epicutaneous application and on intradermical injection, provoke dermatitis characterized by a latent period and cause erythema and pigmentation. Recently Jurlis[39] studied the photo-oxidation of dopa in presence of xanthotoxin under different conditions.

Inspite of these diverse findings the precise mechanisms, whereby furocoumarins function in the treatment of leucoderma is unknown. The biochemical events leading to the formation of melanin, have been clarified, but no clear cut implication of furocoumarins with it has been demonstrated. The present chapter therefore attempts to describe the photo-oxidation of dopa in presence of psoralen using ultraviolet radiation (maximum emission 2537A°) white light (photo flood lamp) and solar irradiation. Studies have also been included on the effect of certain well known inhibitors such as glutathione, cysteine, thiourea, and ascorbic acid.

# METHODS AND MATERIALS

## Reagents

Dihydroxyphenylalanine (DOPA) and glutathione (Sigma chemicals), ascorbic acid (F. Hoffmann-La Roche and Basle) copper sulfate (British Drug Houses, England, A.R.) and cysteine hydrochloride (E.Merck) were made in phosphate buffer of required pH at a concentration of 1 mg/ml. Solution of dopa was prepared as and when required since on storage its auto-oxidation was found to take place. Psoralen solution was prepared in ethyl alcohol in a concentration of 1 mg/ml and stored in brown bottle or in test tube covered with black paper in order to protect it from light. This was diluted before use. Phosphate buffers of different pH were prepared as usual by mixing M/15 $KH_2PO_4$ and M/15 $Na_2HPO_4$. All the reagents were stored in cold until used.

The artificial light sources used in the present studies were as follows:

(a) White light- Photo flood lamp 250 Watt, (Pradip Lamp Works, Patna, India, in collaboration with M/S Tokyo Shibaura Electric Co. Ltd., Japan).

(b) Ultraviolet- Hanovia chromatolite lamp (Model No.30 maximum emission 2537A°.

(c) Solar light (Months of July and August).

## Reaction Mixture

The reaction mixtures normally consisted of a requisite amount of psoralen solution in alcohol, and 1 ml dopa to which phosphate buffer (M/15) of required pH was added to make final volume to 4.0 ml. The reaction mixture before and after irradiation was kept in covered aluminium tubes (size 2 X 6 cm).

## Irradiation Procedure

(a) Ultraviolet irradiation was done in a darkroom at room temperature (25-30°C). The reaction mixture was transferred to 50 ml pyrex beakers which were placed under the ultraviolet lamp kept at a distance of 10 cm from the bottom of the fluid surface. After irradiation the mixture was transferred to the respective tube and kept in cold until the spectra were taken.

(b) Irradiation with photo flood lamp was done in dark room in beakers sorrounded by ice to avoid rise in temperature, samples were placed under the lamp at a distance of 15 cm from the surface of the reaction mixture.

(c) Solar irradiation was done in bright sun in beakers sorrounded by ice for requisite periods.

Absorption spectra in these studies were taken immediately after irradiation on a Beckmann DU spectrophotometer.

## RESULTS

The photo-oxidation of dopa under different conditions of irradiation in presence of psoralen has been graphically presented in Fig. 1 to 6.

From Fig.1, representing the photo-oxidation of dopa irradiated with photo flood lamp, it is clear that psoralen *per se* in the dark or dopa irradiated alone did not bring about any appreciable oxidation, whereas when dopa together with psoralen was irradiated, considerable oxidation of dopa was observed. The absorption spectra of the oxidized product exhibited a peak at 460 mμ which is identical to the peak obtained with dopachrome[9]. Optical density values at 460 mμ of dopa irradiated alone and with psoralen were 0.012 and 0.102 respectively, indicating an increase of 88.53 per cent. The absorption spectra of irradiated psoralen showed a slight absorption, which was found to be more towards the shorter wavelength.

Fig.2 represents the photo-oxidation of dopa when the irradiation was done with short wavelength ultraviolet light (maximum emission 2537A°). In these experiments the concentration of psoralen was varied from 0.05 mg to 1.0 mg and the irradiation was performed for 30 minutes at pH 7.0. From the

Fig. 1 Photo-oxidation of DOPA by Psoralen in white light.

1 - DOPA without psoralen

2 - Psoralen without DOPA

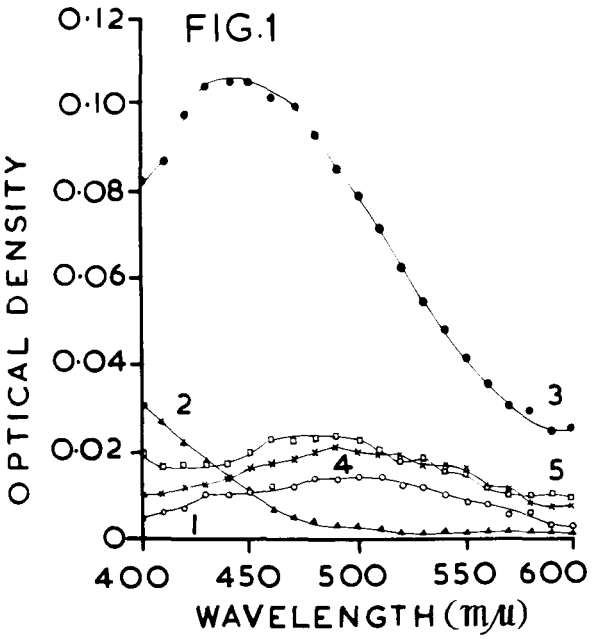
3 - DOPA plus psoralen

4 - DOPA kept in dark

5 - Psoralen plus DOPA kept in dark

Irradiation time - 30 minutes;
Psoralen concentration
1.0 mg/ 4 ml; pH - 7.0

FIG.1


0·12
0·10
0·08
0·06
0·04
0·02
0
OPTICAL DENSITY
400
450
500
550
600
WAVELENGTH (mμ)
1
2
3
4
5

Effect of psoralen concentration on the photo-oxidation of DOPA by ultraviolet light.

Fig.2A
1. DOPA without psoralen
2. Psoralen (concentration 0.25, 0.50 and 1.00 mg/4 ml) without DOPA
3. DOPA plus psoralen (1.0 mg/4 ml)
4. DOPA plus psoralen (0.5 mg/4 ml)
5. DOPA plus psoralen (0.25mg/4 ml)

Fig.2B
1. DOPA without psoralen
2. Psoralen (concentration 0.05, 0.10 and 0.20 mg/4 ml) without DOPA
3. DOPA plus psoralen (0.05 mg/4 ml)
4. DOPA plus psoralen (0.10 mg/4 ml)
5. DOPA plus psoralen (0.20 mg/4 ml)

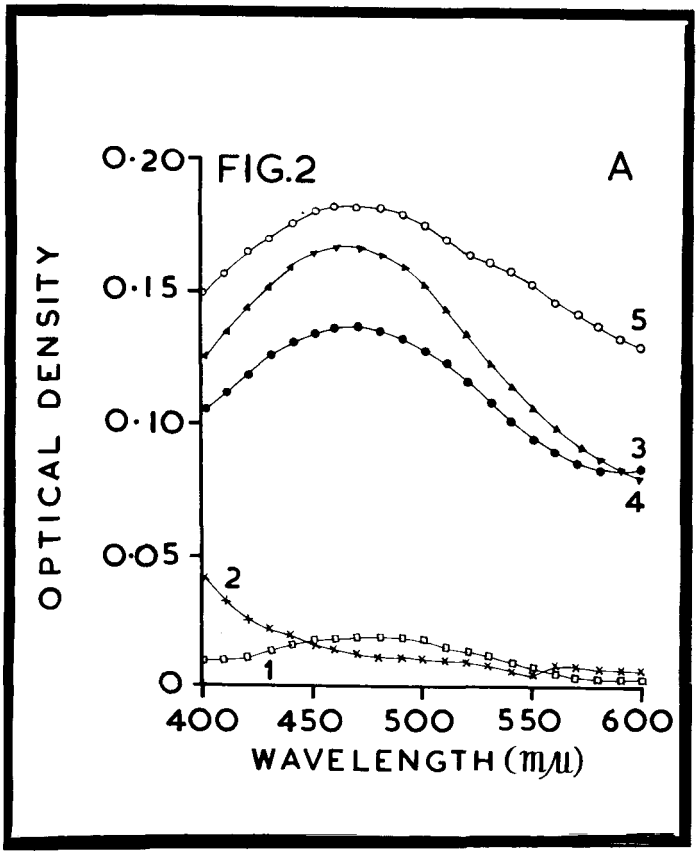


FIG.2
A
0·20
0·15
0·10
0·05
0
OPTICAL DENSITY
5
3
4
2
1
400
450
500
550
600
WAVELENGTH (mμ)

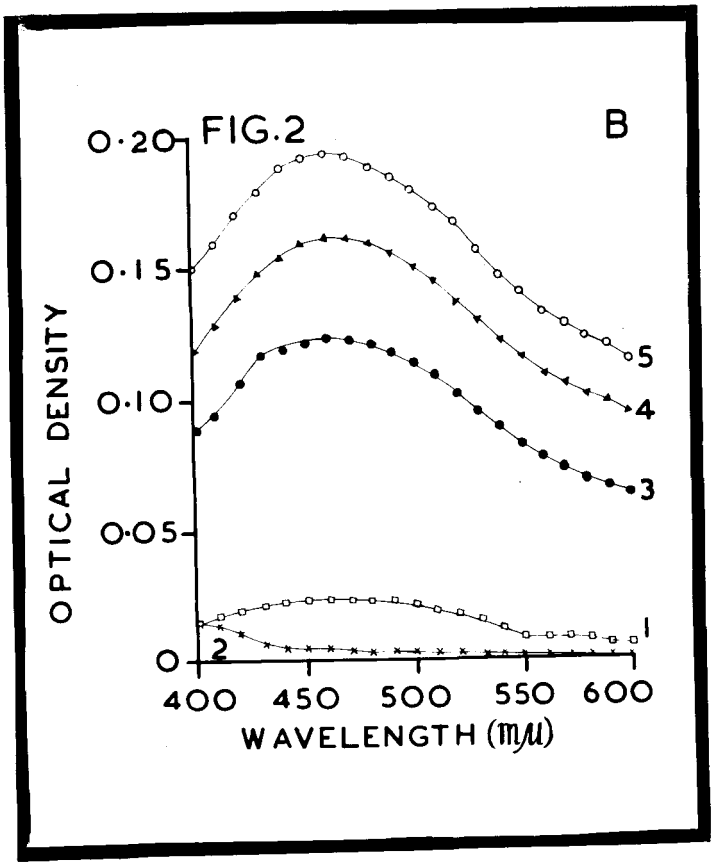


FIG.2
B
0·20
0·15
0·10
0·05
0
OPTICAL DENSITY
5
4
3
1
2
400
450
500
550
600
WAVELENGTH (mμ)

figure it is evident that increase in psoralen concentration from 0.05 mg to 0.2 mg significantly increased the photo-oxidation of dopa. On a further increase in the concentration of furocoumarin upto 1.0 mg the photo-oxidation was seen to fall gradually. With a view to have a better comprehension the values of optical density at 460 mμ with different concentration of psoralen have been ploted in Fig.3, which would again show that photo-oxidation of dopa increases with increasing concentration of psoralen upto 0.20 mg, beyond this concentration a decline was obtained. The photo-oxidation of dopa without psoralen was almost insignificant in all these cases.

The effect of certain -SH compounds, known to be inhibitors of melanin formation, e.g. glutathione, cysteine, thiourea and that of ascorbic acid was studied on the photo-oxidation of dopa by ultraviolet light in presence of psoralen and the results are presented in Fig.4. The data in the figure would show that the photo-oxidation is significantly inhibited by glutathione, cysteine and thiourea. The degree of inhibition was maximum with glutathione, next in order was cysteine followed by thiourea. With cysteine and glutathione the absorption at 460mμ was almost found to disappear and only a generalized absorption getting stronger towards shorter wavelength

Fig. 3 Effect of psoralen concentration on the photo-oxidation of DOPA in ultraviolet light.

(The values represents O.D. at 460mμ)

1. DOPA without psoralen
2. Psoralen without DOPA
3. DOPA plus psoralen

Concentration of psoralen in A, B, C, D, E, and F was 0.05, 0.10, 0.20, 0.25, 0.50 and 1.00 mg per 4.0 ml of the reaction mixture; Irradiation time - 20 minutes; pH - 7.0

FIG.3

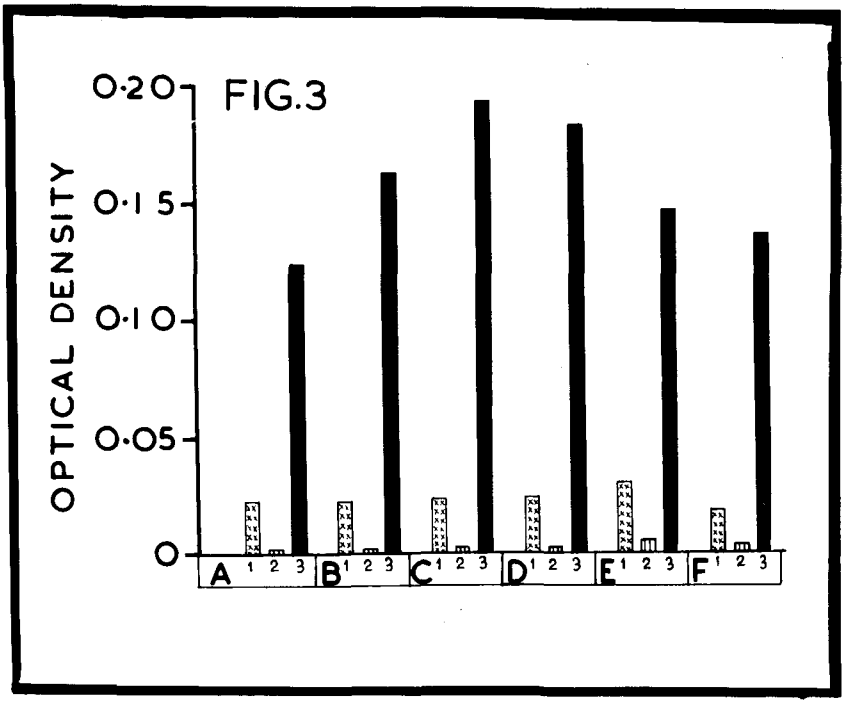

OPTICAL DENSITY
0·20
0·15
0·10
0·05
0
A 1 2 3
B 1 2 3
C 1 2 3
D 1 2 3
E 1 2 3
F 1 2 3

Fig. 4 Effect of -SH compounds on the photo-oxidation of DOPA by psoralen in ultraviolet light.

1. DOPA without psoralen
2. Psoralen without DOPA
3. DOPA plus psoralen
4. DOPA plus thiourea
5. DOPA, psoralen and thiourea
6. DOPA plus glutathione
7. DOPA, glutathione and psoralen
8. DOPA, cysteine and psoralen

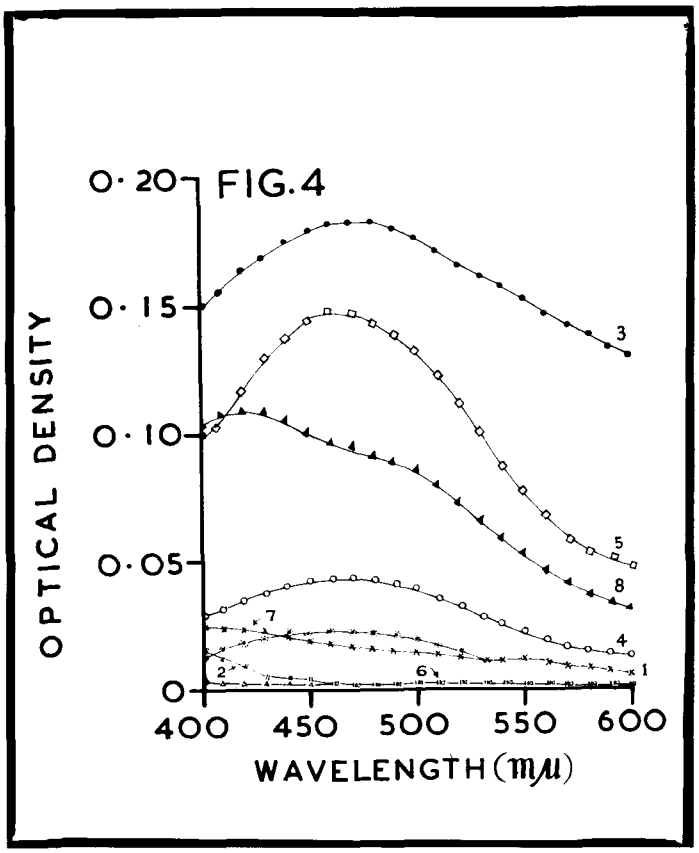


FIG.4
OPTICAL DENSITY
0·20
0·15
0·10
0·05
0
400
450
500
550
600
WAVELENGTH (mμ)
1
2
3
4
5
6
7
8

was obtained. Comparison of OD values at 460 mμ indicate the percentage inhibition to be 19.23, 46.15 and 90.10 with thiourea, cysteine and glutathione. Complete inhibition of the photo-oxidation of dopa was observed when ascorbic acid was added to the reaction mixture.

The photo-oxidation of dopa by solar light in presence of psoralen is represented in Fig.5. Oxidation was studied at pH 7.0 for 6,12 and 18 minutes, with a psoralen concentration of 0.25 mg per 4.0 ml. It may be seen from the figure that solar irradiation per se without psoralen upto 18 minutes did not have any significant photo-oxidation of dopa. However, when dopa was irradiated in presence of psoralen rapid oxidation was found to take place and the absorption spectra, as usual, exhibited a peak at 460 mμ, the OD values at this wavelength at 6, 12 and 18 minutes irradiation being 0.160, 0.303 and 0.341 respectively. The above values would indicate that the extent of photo-oxidation was significantly increased with the period of irradiation upto 12 minutes, after which in the next 6 minutes, the increase was comparatively less.

Studies on the effect of different pH (6.2, 7.0 and 8.0) on the photo-oxidation of dopa in presence of psoralen by solar light (Fig.6) indicate, that pH has almost no effect on either the photo-oxidation of

Fig. 5 Photo-oxidation of DOPA by psoralen in solar light.
(Irradiation time A - 6 min.; B - 12 min.; and C - 18 min.)
1. DOPA without psoralen
2. Psoralen without DOPA
3. DOPA plus psoralen

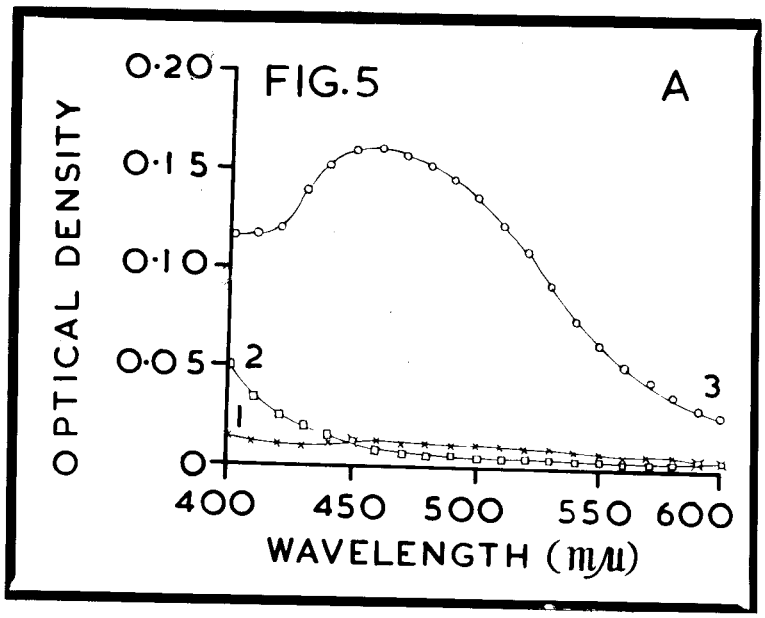


FIG.5
A
OPTICAL DENSITY
0·20
0·15
0·10
0·05
0
1
2
3
400
450
500
550
600
WAVELENGTH (mμ)

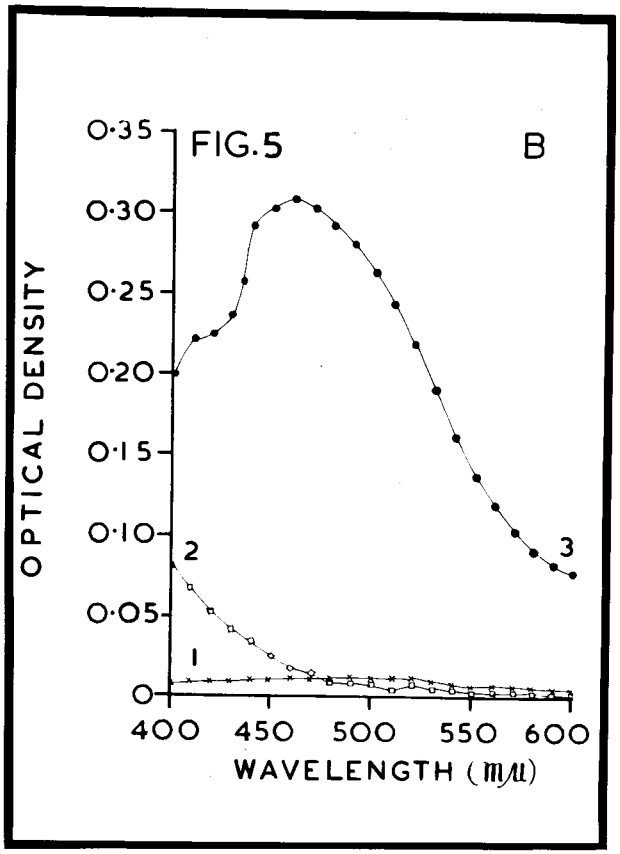


FIG.5
B
OPTICAL DENSITY
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0
1
2
3
400
450
500
550
600
WAVELENGTH (mμ)

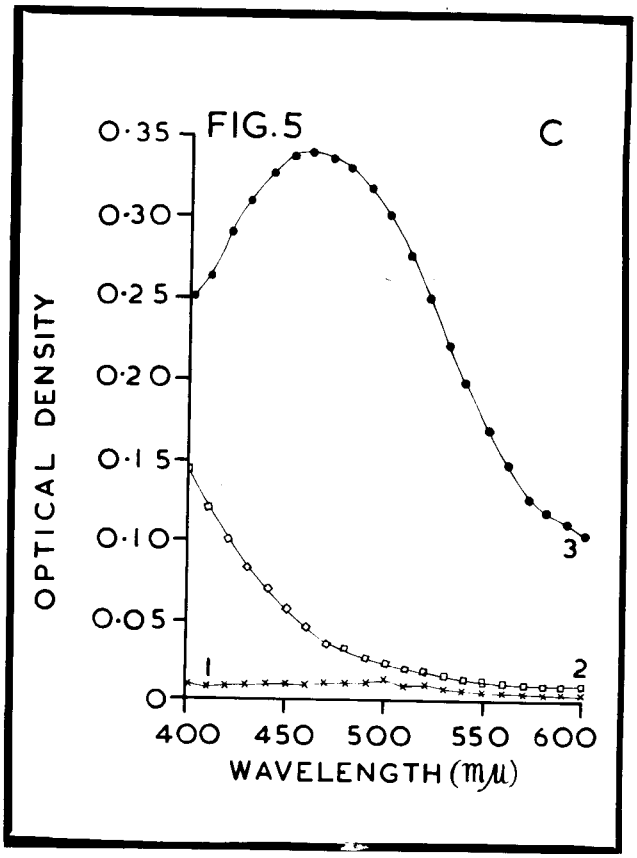


FIG.5
C
OPTICAL DENSITY
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0
1
2
3
400
450
500
550
600
WAVELENGTH (mμ)

Fig. 6 Effect of pH on the photo-oxidation of DOPA by solar irradiation.
( A - pH 6.2; B - pH 7.0; and C - pH 8.0)

1. DOPA without psoralen
2. Psoralen without DOPA
3. DOPA plus psoralen

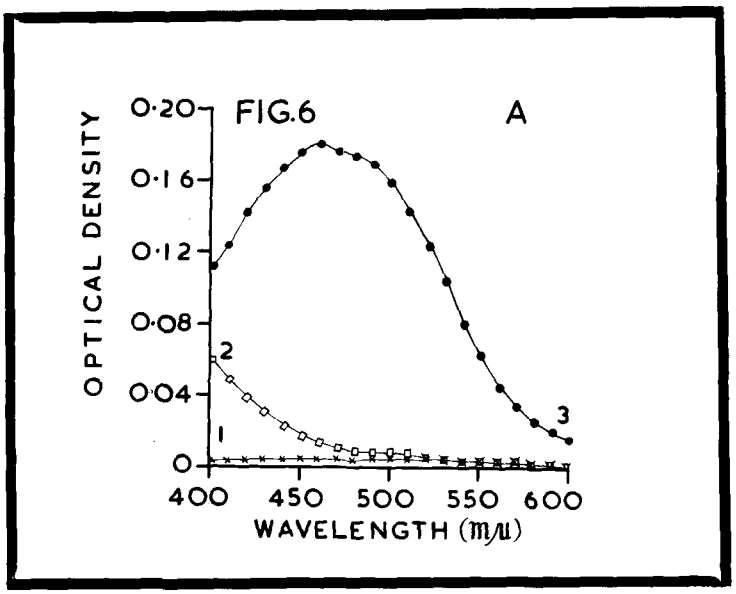


FIG.6
A
OPTICAL DENSITY
0·20
0·16
0·12
0·08
0·04
0
1
2
3
400
450
500
550
600
WAVELENGTH (mμ)

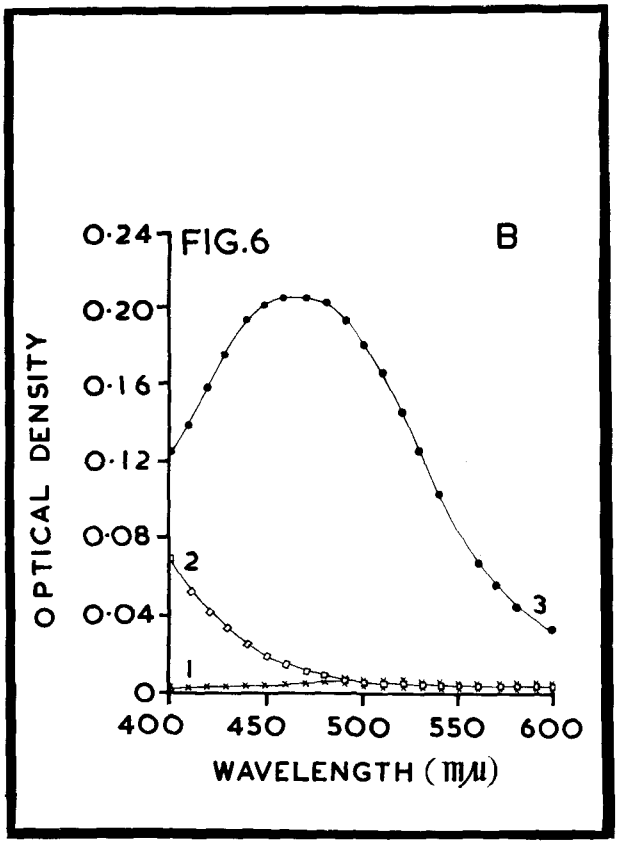


FIG.6
B
OPTICAL DENSITY
0·24
0·20
0·16
0·12
0·08
0·04
0
1
2
3
400
450
500
550
600
WAVELENGTH (mμ)

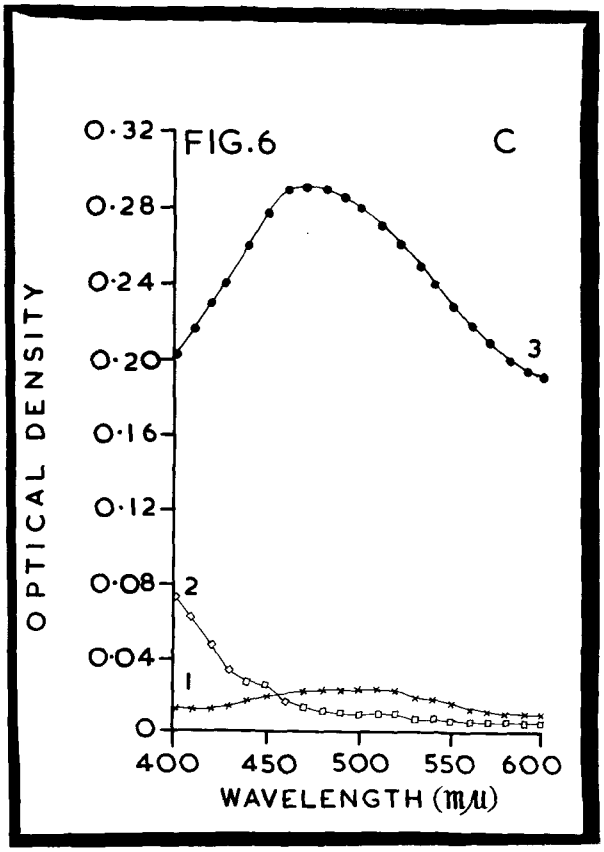


FIG.6
C
OPTICAL DENSITY
0·32
0·28
0·24
0·20
0·16
0·12
0·08
0·04
0
1
2
3
400
450
500
550
600
WAVELENGTH (mμ)

dopa per se or that of psoralen alone. However, in presence of psoralen the photo-oxidation of dopa was significantly affected by a change in the hydrogen ion concentration of the reaction mixture and it was seen to increase with the increase of pH, although the pattern of absorption spectra did not change. At pH 6.2, the OD value at 460 mμ was 0.198 and it increased to 0.204 and 0.292 at pH 7.0 and 8.0 respectively, which would roughly indicate an increase of 62% in the amount of pigment at pH 8.0 as compared to pH 6.2.

## DISCUSSION

The results of the present study would show that presence of psoralen significantly accelerates the photo-oxidation of dopa under a variety of irradiation conditions, although the actual amount of pigment formed with the short wavelength ultraviolet light and white light was considerably lower as compared to that obtained with solar light. In this connection it would be interesting to point out that long wavelength ultraviolet light (3200-3400A°) has long been known to potentiate the response of the skin (erythema and sun-tanning) to the action of furocoumarins[40,41]. The results are essentially in accordance with the above findings. Solar irradia-

tion has given the maximum pigment in the presence of psoralen and essentially such radiations are known to have a considerable proportion of long wavelength ultraviolet light[42]. But there has also been a significant increase in the photodynamic action of psoralen in short wave ultraviolet light and may indicate its possible role in pigment production.

The increase of photo-oxidation of dopa with increasing concentration of psoralen upto a certain maximum is easily understood and can be readily explained on the basis of stoichiometric requirements of the photosensitizer in a radiation induced reaction. Excess molecules of psoralen will therefore not function in bringing about an increased pigmentation. However, the decrease in photo-oxidation at very high concentration may be due to the screening effect of the furocoumarin _per se_ as has been observed for other photosensitizers[43-45] or it may be that at higher concentration, dimerization or other chemical reactions take place and the altered molecules are unable to function as photosensitizer in bringing about the oxidation of dopa.

The increased photo-oxidation of dopa at higher pH in the present studies is almost similar to that observed by Judis when xanthotoxin was used as the photosensitizer. Hirsch[46] also found better auto-

oxidation of dopa and consequently melanin yields, with increase in pH. Mosher et al.[47] have further shown that auto-oxidation of dopa is catalysed by hydroxyl ions. He proposed a hypothesis to account for the observed effect of hydroxyl ions. Recently Snell[48] studying the effect of acid and alkali on melanogenesis in the skin of male guinea pigs, found that acetic acid caused a reduction in the depth of colour of the melanin with melanocytes of skin and anterior abdominal wall, together with an increase in the length and complexity of the dendritic processes. The amount of free melanin although unchanged, the number of melanocytes was seen to be augmented. Sodium hydroxide on the other hand caused an increase in the amount of free melanin.

The observed inhibition in the production of pigment from psoralen catalysed photo-oxidation of dopa by -SH compounds e.g. cysteine, glutathione and thiourea may be due to the fact that such compounds are known to combine with the intermediate products in the melanin reaction, leading thereby to lesser pigmentation. Such observation have been made by Roston[49] and Kohn[50], who have indicated the intermediary stages at which sulfhydryl compounds are seen to combine.

The inhibition in the photo-oxidation of dopa by ascorbic acid may once again be due to its reducing properties[51,52], although no chemical combination, as observed with -SH compounds, has so far been reported.

It is well known that in the normal sun-tanning process, melanin formation is stimulated[3,9]. The mechanism by which furocoumarins may stimulate the sun-tanning process could possibly be due to its accumulation in the melanocytes where it would photo-oxidize the available dopa to melanin, giving rise to increased melanin formation.

## SUMMARY

The photo-oxidation of dopa has been studied in presence of psoralen using different kinds of radiations. Sunlight, white light (photo flood lamp) and short wavelength ultraviolet light (maximum emission 2537A°) were found to stimulate the oxidation of dopa in presence of psoralen but not in its absence. The amount of pigment formed with the short wavelength ultraviolet light and white light was considerably less as that obtained with sunlight. The concentration of psoralen was varied from

0.05 mg to 1.0 mg when ultraviolet was used. Maximum photo-oxidation was obtained at a concentration of 0.2 mg per 4.0 ml of the reaction mixture. Further increase in psoralen concentration was observed to decrease the extent of oxidation and thus pigment formation. Studying the effect of certain -SH compounds, it was found that the oxidation was considerably inhibited by such compounds. Complete inhibition of the photo-oxidation was observed when ascorbic acid was added to the reaction mixture. The photo-oxidation of dopa was also significantly affected by a change in the hydrogen ion concentration in the reaction mixture and was seen to be enhanced with the increase of pH.

REFERENCES


1. Bloch, B., Z.Physiol.Chem., 33 (1916) 226.

2. Bloch, B., Das Pigment.In:Jadassohn, Handb.d.Haut-u. Geschlechtskr., 1/1 434-51. Berlin:J.Springer, 1927.

3. Bloch, B. and Schaaf, F., Biochem.Z., 182 (1925) 181.

4. Mazza, F.P. and Stolfi, G., Brit.J.Dermatol., 52 (1940) 205.

5. Moncorps, C., — Arch.Dermatol.Syph., 148 (1934) 1.

6. Schaaf, F., — Ibid., 178 (1938) 648.

7. Lerner, A.B., — Am.J.Med.,19 (1955) 902.

8. Levin, H.M., — Quart.Bull.Northwestern Univ.Med.School, 30 (1956) 1.

9. Fitzpatrick, T.B. and Szabo, G., — J.Invest.Dermatol., 32 (1959) 197.

10. Lerner, A.B. and Case, J.D., — Ibid., 32 (1959) 211.

11. Lerner, A.B., Fitzpatrick, T.B., Calkins, E. and Summerson, W.H., — J.Biol.Chem., 197 (1950) 793.

12. Kubowitz, F., — Biochem.Z., 299 (1937) 221.

13. Idem — Ibid., 299 (1938) 32.

14. Lerner, A.B. and Fitzpatrick, T.B., — Physiol.Rev., 30 (1950) 91.

15. Rothman, S., Krysa, H.F. and Smiljanic, A.M., — Proc.Soc.Exp.Biol.Med., 62 (1946) 209.

16. Lerner, A.B., Shizume, K. and Bunding, I., — J.Clin.Endocrinol.Metab., 14 (1954) 1463.

17. Lerner, A.B., — Ann.Rev.Med., 11 (1960) 197.

18. McGuire, J.S. and Lerner, A.B., — Ann.N.Y.Acad.Sci., 100 (1963) 622.

19. Flege, F.H.J., — J.Cellular Comp.Physiol., 15 (1940) 233.

20. Musajo, L. and Rodighiero, G., — Experientia, 18 (1962) 153.

21. Soine, T.O. Abu-Shady, H. and DiGangi, F.E., — J.Pharm.Sci., 45 (1956) 425.

22. Musajo, L., Rodighiero, G. and Caporale, G., — Bull.Soc.Chim.Biol., 36 (1954) 1213.

23. Musajo, L., Rodighiero, G., Caporale, G. and Antonello, C., — Il Farmaco Ed.Sci., 13 (1958) 355.

24. Caporale, G., — Ibid., 13 (1958) 794.

25. Pathak, M.A. and Fitzpatrick, T.B., — J.Invest.Dermatol., 32 (1959) 255, 509.

26. Pathak, M.A., Fellman, J.H. and Kaufman, K.D., — Ibid., 35 (1960) 165.

27. Fahmy, I.R. and Abu-Shady, H., — Quart.J.Pharm.Pharmacol., 20 (1947) 291.

28. El-Mofty, A.M., — J.Por.Egypt.Med.Assoc., 31 (1948) 651.

29. Idem — Brit.J.Dermatol., 76 (1964) 56.

30. Fowlks, W.L., — J.Invest.Dermatol., 32 (1959) 233.

31. Pathak, M.A. and Fowlks, W.L., — J.Invest.Dermatol., 37 (1961) 183.

32. Fowlks, W.L., Griffith, D.G. and Oginsky, E.L., — Nature, 181 (1958) 571.

33. Oginsky, E.L., Green, G.R., Griffith, D.G. and Fowlks, W.L., — J.Bacteriol., 78 (1959) 821.

34. Musajo, L. and Rodighiero, G., — Nature, 190 (1961) 1109.

35. Pathak, M.A. and Fellman, J.H., — Ibid., 185 (1960) 392.

36. Rodighiero, G. and Bergamasco, C., — II Farmaco Pavia,Ed.Sci., 13 (1958) 369.

37. Rodighiero, G. and Caporale, G., — Ibid., 13 (1958) 373.

38. Fornasari, E. and Rodighiero, G., — Ibid., 13 (1958) 379.

39. Joseph Judis, — J.Am.Pharm.Assoc., 49 (1960) 447.

40. Soine, T.O., — J.Pharm.Sci., 53 (1964) 231.

41. Imbrie, J.D., Daniels, F.(Jr), Bergeron, L., Hopkins, C.E. and Fitzpatrick, T.B., — J.Invest.Dermatol., 32 (1959) 331.

42. Pathak, M.A., Riley, F.J., Fitzpatrick, T.B. and Curwen, W.L., — Nature, 193 (1962) 148.

43. Heinmets, F., Vinegar, R. and Taylor, W.W., — J.Gen.Physiol., 36 (1952) 207.

44. Weil, L. and Maher, J., — Arch.Biochem., 29 (1950) 241.

45. Welsh, J.N. and Adams, M.H., — J.Bacteriol., 68 (1954) 122.

46. Hirsch, H.M., — 'Pigment Cell Biology' Academic Press Inc.,New York, N.Y., 1959 p.330.

47. Mender, C., Williams, J.N.(Jr) and Waisman, H.A., — Arch.Biochem.Biophys., 72 (1957) 255.

48. Snell, E.S., — Z.Zellforsch.Abt.Histochem., 57 (1962) 376.

49. Roston, S., — J.Biol.Chem., 235 (1960) 1902.

50. Kohn. H.R., — Enzymologia, 17 (1954-56) 193.

51. Visetti, M. and Ferrero, F., — Minerva Dermatol., 2 (1957) 457.

52. Scharf, W. and Dawson, C.R., — J.Am.Chem.Soc., 80 (1958) 4627.

53. Daniels, F.(Jr), — J.Invest.Dermatol., 32 (1959) 147.

# CHAPTER V

## IN VIVO STUDIES IN RELATION TO GROWTH, ORGAN WEIGHT AND INCORPORATION OF $P^{32}$ IN ALBINO RATS.

Considerable work has been done on the clinical aspects of furocoumarins which are known to potentiate the action of ultraviolet light in pigment production[1-3]. Thus Lerner et al.[4] used methoxsalen orally in nine vitiligo patients with moderate success. Fitzpatrick and Lerner[5] used methoxsalen orally in the treatment of 110 patients and extended the testimonial type of evidence of increased sun tolerance in vitiligo skin following treatment. El-Mofty[6] found the minimum lethal dose (MLD) in mice for 8-methoxypsoralen and 8-isoamyleneoxypsoralen was 300 mg/kg and 330 mg/kg respectively, whereas for guinea pigs Elvi[7] reported an MLD of 400 and 800 mg/kg respectively for the same compounds. Studying the toxicity, Mukherji[8] has shown that feeding of psoralen to albino rats resulted in 30 per cent increase in the weight of liver, while the weight of spleen became double. The present chapter describes the effect of prolonged psoralen feeding, with and without simultaneous ultraviolet irradiation in relation to growth, organ weight and incorporation of radioactive phosphorus in different organs of albino rats.

## METHODS AND MATERIALS

Male albino rats of about 100 gm weight from Central Drug Research Institute Colony, were divided into the following four groups of six rats each and were housed separately in different cages:-

Group I - Normal

Group II - Rats fed with psoralen.

Group III - Normal irradiated.

Group IV - Psoralen fed and irradiated.

All animals were maintained under uniform laboratory conditions throughout the experimental period.

### Administration of drug

Psoralen was well ground in an all glass pestle and mortar before administration. The composition of the feeding mixture for each group was as follows:-

### Control

250 mg gum tragaconth, 750 mg of glucose and 2.5 ml of ethyl alcohol, rest water to make 250 ml

### Psoralen

250 mg gum tragaconth, 750 mg glucose, 125 mg psoralen and 2.5 ml ethyl alcohol, rest water to make 250 ml.

Solutions were administered by a feeding needle, having a rounded tip. Each rat was fed with 0.5 ml of the respective solution while in the animals of group III and IV, the administration of solutions was followed by irradiation with ultraviolet lamp. For irradiation the rats were shaved at the back using clippers. Shaving was repeated at weekly intervals.

Ultraviolet irradiation

Ultraviolet irradiation was carried out in the dark room at room temperature (22-27°C) using a Hanovia chromatolite lamp with a filter attachment to emit most of the radiation at 2537A°. Rats to be irradiated were kept in small separate cages, which were kept underneath the lamp at a distance of 15 cm from the surface on which the cage was placed. Two rats, one control and one psoralen fed, were irradiated at a time. Radiation was carried out after two to three hours of feeding of psoralen. Each rat was given a radiation dose of two minutes.

Incorporation of radioactive phosphorus

Feeding of rats of group I and II was done for 92 days while that of group III and IV for 116 days. The rats were then given a single dose (10 microcurie/ 100 gm of body weight) of $P^{32}$ (as orthophosphate in isotonic saline solution with phosphate buffer pH 7.0,

sterilized) in the right femur region, intramuscularly. Two hours after injection of $P^{32}$, the animals were sacrificed by rupture of the spinal cord. Organs (liver, kidney, heart, spleen, lungs, muscle, brain and skin) were quickly removed and blotted on a filter paper, and weighed. They were then digested in pyrex tubes with Conc. $HNO_3$(AR). Five ml of the acid was added in each case (weight of tissue from 1 to 2 gm) and the digestion was carried out in boiling water bath. After digestion, the clear solutions of the different organs were diluted with distilled acetone (in order to dissolve the remaining fat) to a known volume. Five ml aliquots were counted in a sleeve type of G.M. counter with a requisite scaler and timer unit (fabricated at the Atomic Energy Establishment Trombay). For control a known amount of the original $P^{32}$ solutions, was diluted to a known amount and five ml were counted. From this the dose of $P^{32}$ injected was calculated in terms of exact count given to a rat. The uptake of radiophosphorus was then calculated per gm of the wet tissue. The results have been presented as counts per minute (cpm) when 10,000 cpm were given per 100 gm of body weight. Standard deviation has also been calculated for each set. Assay of radioactivity and calculation were done essentially according to the procedure described earlier[9].

# RESULTS

## Effect of psoralen on growth of rats

The results of the present study would show that feeding of psoralen to rats for a period of nine weeks (with and without ultraviolet irradiation) did not appreciably bring about any change in the growth of rats (Table I).

Table I - Effect of feeding psoralen (upto nine weeks) with and without irradiation on growth of rats *.

| Weight (gm) | Non-irradiated | | Irradiated | |
|---|---|---|---|---|
| | Control | Psoralen | Control | Psoralen |
| Initial Wt. | 83.00 | 83.33 | 149.13 | 144.00 |
| ± S D | ± 11.82 | ± 4.32 | ± 16.12 | ± 23.55 |
| Final Wt. | 137.30 | 136.30 | 192.83 | 201.00 |
| ± S D | ± 13.25 | ± 13.38 | ± 21.03 | ± 25.77 |

* Values given are mean of six animals.

Control rats on animal house diet registered an increase in weight of approximately 54 gm in nine weeks, whereas when psoralen was simultaneously fed, the nett increase in weight was found to be 53 gm. However in the irradiated group the control animals per se increased in weight by only 44 gm which is less than in the control animals.

Administration of psoralen to this group, not only restored the weight, but the animals actually were found to grow better and the gain in weight in nine weeks was 57 gm.

Plotting the increase in weight against time (Fig. 1 & 2) it was seen that in the non-irradiated group a uniform increase in weight was observed both in normal and psoralen fed animals. On the contrary in the irradiated group, a decline in growth was observed during the first two weeks, later being followed by an increase, both in normal and psoralen fed animals (Fig.2), which is in agreement with those reported by Blum et al.[10]

Effect of feeding psoralen on the weight of different organs.

With a view to find out any deleterious effect or otherwise on specific organs, the effect of feeding psoralen to irradiated and non-irradiated animals was studied with respect to the weight of kidney, heart, spleen and brain (Table II). As is evident, feeding of psoralen per se without ultraviolet irradiation did not bring about any change in the weight of kidney, heart and brain. However, the weight of spleen was significantly enhanced, its increase being almost 36 per cent above that of the control animals; the exact weight being 0.249 gm in control and 0.339 gm in psoralen fed animals. In rats

Fig. 1 Effect of feeding psoralen on the growth of rats.

A - Control

B - Psoralen fed

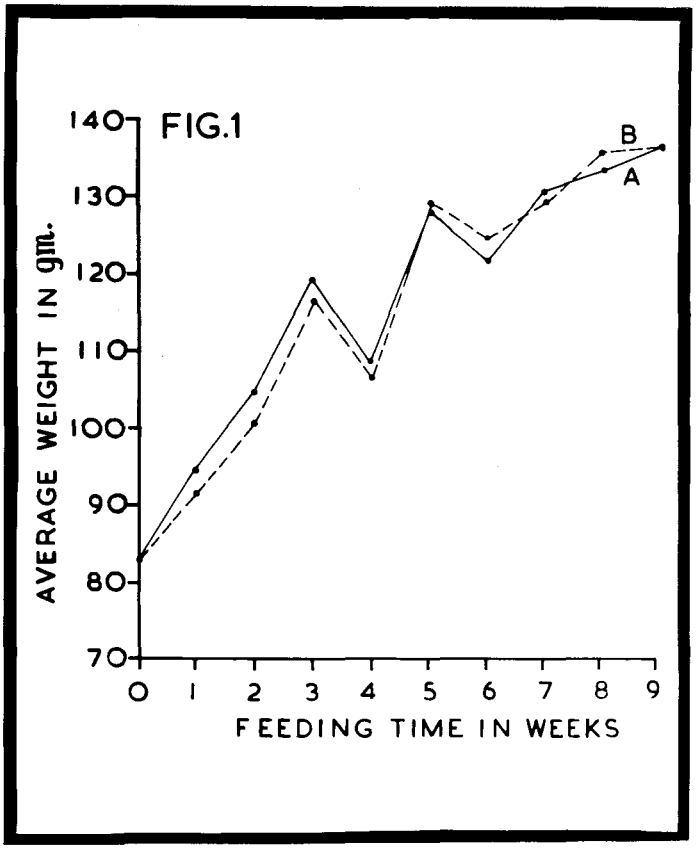


FIG.1
140
130
120
110
100
90
80
70
AVERAGE WEIGHT IN gm.
0 1 2 3 4 5 6 7 8 9
FEEDING TIME IN WEEKS
B
A

Fig. 2 Effect of feeding psoralen (with simultaneous ultraviolet irradiation) on the growth of rats.

A - Control

B - Psoralen fed

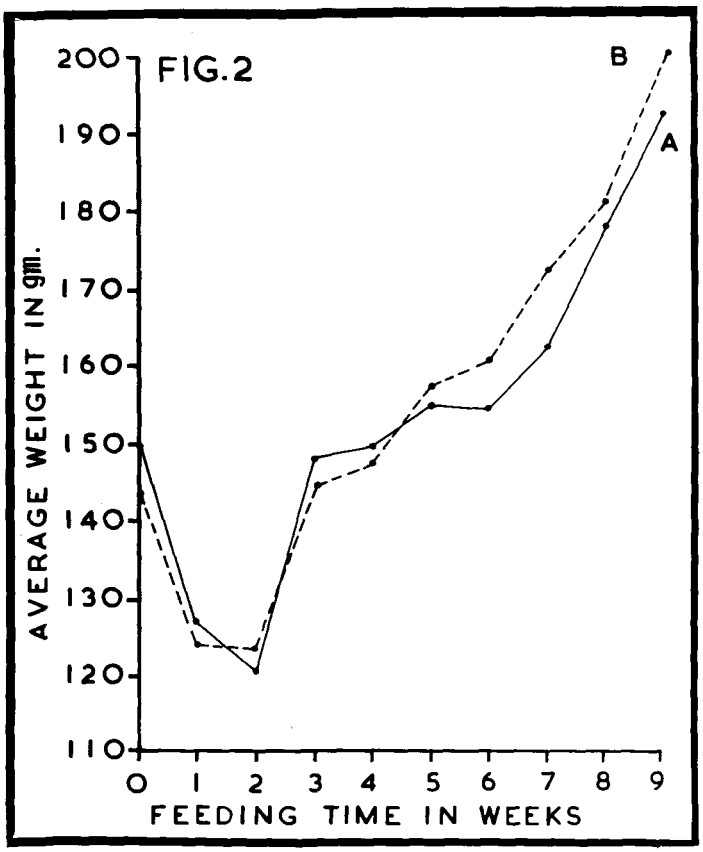


FIG.2
B
A
200
190
180
170
160
150
140
130
120
110
AVERAGE WEIGHT IN gm.
0 1 2 3 4 5 6 7 8 9
FEEDING TIME IN WEEKS

fed with psoralen with subsequent irradiation, the weight of all the four organs including spleen was found to be unchanged.

Table II - Weight* of different organs of psoralen fed rats with and without ultraviolet irradiation.

| Organs | Non-irradiated | | Irradiated | |
|---|---|---|---|---|
| | Control | Psoralen | Control | Psoralen |
| Kidney | 0.338 ± 0.013 | 0.327 ± 0.050 | 0.321 ± 0.055 | 0.313 ± 0.059 |
| Heart | 0.344 ± 0.022 | 0.334 ± 0.031 | 0.298 ± 0.019 | 0.295 ± 0.020 |
| Spleen | 0.349 ± 0.015 | 0.338 ± 0.020 | 0.255 ± 0.059 | 0.251 ± 0.040 |
| Brain | 0.822 ± 0.175 | 1.187 ± 0.101 | 0.796 ± 0.152 | 0.776 ± 0.110 |

*Weights of organs have been expressed as per cent of body weight.

Standard deviation has been indicated after each value. Number of animals used in this study were six.

## $P^{32}$ incorporation

The relative distribution of radioactivity per gm wet weight of different tissues in normal and psoralen fed rats with and without simultaneous ultraviolet irradiation, presented in Table III, would show that amongst the different organs assayed for radioactivity, in normal animals, the injected phosphorus attains a greater concen-

tration in liver and kidney as compared to heart, lung, muscle, skin and brain. The lower incorporation in brain may probably be due to the presence of blood brain barrier. As is evident from the table, feeding psoralen with and without ultraviolet irradiation was not found to have any significant effect on the incorporation of $P^{32}$ in the different organs.

Table III - Incorporation of radioactive phosphorus $P^{32}$ in normal and psoralen fed rats with and without ultraviolet irradiation.

| Organs | Non-irradiated | | Irradiated | |
|---|---|---|---|---|
| | Control | Psoralen | Control | Psoralen |
| Liver | 319 ± 46 | 312 ± 58 | 269 ± 60 | 223 ± 51 |
| Kidney | 179 ± 32 | 133 ± 22 | 154 ± 19 | 146 ± 27 |
| Heart | 145 ± 29 | 163 ± 34 | 134 ± 9 | 117 ± 28 |
| Spleen | 110 ± 6 | 125 ± 34 | 97 ± 7 | 80 ± 18 |
| Lung | 90 ± 16 | 98 ± 10 | 71 ± 16 | 77 ± 16 |
| Muscle | 94 ± 63 | 77 ± 25 | 101 ± 68 | 180 ± 73 |
| Brain | 9 ± 2 | 9 ± 1 | 8 ± 5 | 8 ± 1 |
| Skin | 29 ± 5 | 32 ± 13 | 31 ± 19 | 29 ± 20 |

The results are given in counts per minute. Dose given 10,000 CPM per 100 gm body weight.
Standard deviation has been indicated after each readings. The data represents mean of six animals.

## DISCUSSION

The results of the present study would show that feeding of psoralen upto nine weeks with and without simultaneous ultraviolet irradiation did not have any effect on either the growth of rats or the increase in weight of different organs. These results are in agreement with those reported by Mukherji who also reported that feeding of some furocoumarins to albino rats upto a period of 10 weeks did not have any effect on the growth. However, he observed an increase in the weight of liver and spleen to the extent of 20 and 100 per cent respectively. In the present studies an increase in the weight of spleen was only about 36 per cent. In the group fed with psoralen and irradiated, the weight of spleen was not affected. This was found in all six animals and is yet difficult to explain. However, it may be pointed out that irradiation and feeding may not be as toxic as the psoralen itself.

From the present studies it would appear that psoralen (2.5 mg/day) with and without simultaneous ultraviolet irradiation, has almost no effect on the growth of rats. This finding is in close agreement to one reported by Lerner[4] that a 15 months old child could tolerate 10 mg of 8-MOP daily for months without

any adverse effect. An adult on the other hand was reported to tolerate a daily dose of 50 mg. In yet another case toleration could not exceed beyond 20 mg/day without suffering from nausea, epigastric distress, increased nervous tension and diarrhoea. El-Mofty[3] and also Lerner[4] consider that 8-MOP administered at a daily dose level of 50 mg is safe for human adult. Mukherji[9] on consideration of surface area, showed that a rat weighing 100 gm should be able to tolerate a daily dosage of about 1.9 mg of 8-MOP, however he used 2.5 mg per rat.

The present studies would further indicate that the incorporation of radioactive phosphorus in different organs was also not affected by the administration of psoralen, with and without ultraviolet irradiation. As the incorporation of phosphorus is an index of nucleic acid synthesis[11,12], it would perhaps be said that synthesis of nucleic acid was not impaired with the feeding of psoralen *per se* or when it was administered simultaneously with ultraviolet irradiation.

## SUMMARY

Feeding of psoralen to rats for nine weeks (with and without simultaneous ultraviolet irradiation, maximum emission 2537A°) was not found to bring about any appreciable change in the growth of rats. In the

irradiated group the animals were actually seen to grow better. Feeding of psoralen *per se* without ultraviolet irradiation did not bring any change in the weight of kidney, heart and brain, however, the weight of spleen was significantly enhanced. In rats fed with psoralen with subsequent irradiation, the weight of all the four organs including spleen was found to be unchanged. Furthermore, feeding psoralen with and without ultraviolet irradiation was once again not found to have any significant effect on the incorporation of $P^{32}$ in different organs.

REFERENCES


1. El-Mofty, A.M., J.Roy.Egypt.Med.Assoc., 35 (1952) 1.
2. Kanof, N.B., J.Invest.Dermatol., 24 (1955) 5.
3. Fitzpatrick, T.B., Hopkins, C.E., Blickenstaff, D.D. and Swift, S., Ibid., 25 (1955) 187.
4. Lerner, A.B., Denton, C.R. and Fitzpatrick, T.B., Ibid., 20 (1953) 299.
5. Fitzpatrick, T.B. and Pathak, M.A., Ibid., 32 (1959) 229.
6. El-Mofty, A.M., J.Roy.Egypt.Med.Assoc., 31 (1948) 651.
7. Elwi, A.M., Ibid., 33 (1950) 773.

8. Mukherji, A., J.Sci.Ind.Res.(India), 12C (1960) 223.

9. Sarkar, T.A., Agarwala, S.C. and Mukerji, B., Bull.Nat.Inst.Sci.(India), No.13 (1962) 332.

10. Blum, H.F., Grady, H.G. and Kirby-Smith, J.S., Am.J.Physiol., 138 (1943) 378.

11. Hanawalt, P. and Setlow, R., Biochim.Biophys.Acta, 41 (1960) 283.

12. Hanawalt, P., Science, 130 (1959) 386.

# CHAPTER VI

## IN VITRO AND IN VIVO EFFECT OF NORMAL AND IRRADIATED PSORALEN ON GLUCOSE OXIDATION OF BRAIN AND LIVER HOMOGENATE.

Using tritium labelled 8-methoxypsoralen, Griffin[1] was able to observe measurable amounts of radioactivity in skin and brain of albino mice, indicating that intraperitoneally injected methoxsalen or its metabolites do appear in these tissues. As indicated in an earlier Chapter III, one of the roles of psoralen in the skin may be that its irradiated products may be inactivating the -SH groups and thereby helping the tyrosinase action, leading to better pigmentation, although Lerner[2] did not observe any effect of Methoxsalen per se on skin tyrosinase. However the exact role of this furocoumarin in the brain is far from being understood, although pituitary is considered to be in vivo regulator of pigment production[3]. It would be interesting in this connection to mention the work of Kitagawa[4] on the effect of coumarin derivatives on the respiration of rat brain tissue. Acceleration of endog-nous oxygen uptake was observed by him at low concentrations

where as higher concentration (10-100 mg per cent) of such derivatives were found to have an inhibitory effect. Further[5] this suppressive action was explained on the basis of cytochrome oxidase inhibition in conformity with the general proposal of Michaelis and Quastel[6] relating to hypnotics and anaesthetics.

In addition to these studies Mofty et al.[7] have shown that 8-MOP and 8-Isoamyleneoxypsoralen have been found to cause no variation in the blood sugar level, when given in doses of 200, 400 and 800 mg per kg of the body weight in acute experimentation. Lerner[2] and Elvi[8] have presented evidence of liver necrosis by the administration of psoralen. In our Institute Mukherji[9] has shown that psoralen when given to rats in a dosage of 2.5 mg/100 gm/day resulted in congestion and enlargement of liver and a 20 per cent increase in its weight.

In view of these observations, it was considered of interest to investigate the effect of normal and irradiated psoralen on the in vitro and in vivo oxidation of glucose in brain and liver of albino rats. The results of such an study are reported in the present chapter.

# METHODS AND MATERIALS

## Irradiation of psoralen

Psoralen solution in 1:1 alcohol was irradiated (Solar) at different periods (1/2 to 6 hours), and was concentrated under an infra-red lamp (Philips, Infra-phil, 150 Watt). The over all procedure were essentially the same as described earlier in Chapter III.

## Reagents

Krebs Ringer solution was prepared according to Umbriet, Burris and Stauffer[9]. M/10 glucose was prepared by dissolving requisite amount of glucose (BDH, Analar) in distilled water. All the solutions were kept in cold, until used.

## Preparation of homogenates

Healthy male albino rats from the Central Drug Research Institute Colony weighing from 80 to 100 gm were taken for these studies. Rats were killed by breaking the spinal cord, and liver and brain were immediately removed, dried on a filter paper, weighed and were chilled in a petri dish dipped in ice bath. The homogenate (20 per cent

weight/volume) was prepared in ice cold Krebs Ringer phosphate buffer pH 7.4 using Potter Elvehjem homogenizer, which was previously cooled in a freezing mixture.

Manometry

Conventional Manometry was used for studying the oxygen uptake in the presence of glucose. A uniform shaking rate of 100 strokes per minute and a constant temperature of 37±0.1°C was maintained throughout the experiment. Each flask contained 1.0 ml of doubly concentrated Krebs Ringer phosphate buffer, 1.0 ml of homogenate and 0.5 ml of concentrated irradiated psoralen (1.5 mg/0.5 ml) in the main compartment, 0.3 ml of glucose in the side arm and 0.2 ml of 10 per cent KOH solution in the center cup with 2 sq cm filter paper to absorb carbon dioxide produced during the oxidation. Total volume in the flasks was always made upto 3.2 ml by adding distilled water.

The flasks were equilibrated for 10 minutes and subsequently the substrate was tipped into the main compartment. Readings were taken at an interval of 30 minutes. Control flasks without irradiated psoralen were also included in each experiment.

Average readings from two flasks were calculated in each case.

## In vivo studies

Male albino rats of about 100 gm weight from Central Drug Research Institute Colony were taken and divided into two groups of six rats each and were housed separately in different cages. One group was kept as control while the other as experimental. All the animals were maintained under uniform laboratory conditions throughout the experimental period. One of the groups was fed with the respective solutions required for control animals and the other was given psoralen. Details of solutions administered is given in Chapter V. Feeding was continued for a week, after which the rats were killed and the oxidation of glucose in both brain and liver was followed as described earlier in the present chapter.

## RESULTS

### In vitro effect of psoralen suspension on Glucose oxidation by brain and liver homogenate.

Effect of psoralen suspension on the *in vitro* metabolism of glucose in liver and brain homogenate was studied, and the results are presented in Table I. As is evident from the table, psoralen suspension had no inhibitory effect on the glucose oxidation by brain and liver homogenates in a period of 120 minutes. On the contrary, slight activation of the glucose oxidation was observed in both brain and liver homogenates.

Table I - Effect of psoralen suspension on the metabolism of glucose in rat brain and liver homogenates.

| Time (minutes) | Oxygen consumed (µl.) | | | |
|---|---|---|---|---|
| | Brain | | Liver | |
| | Control | Psoralen | Control | Psoralen |
| 30 | 85.98 | 87.72 | 88.42 | 97.97 |
| 60 | 134.26 | 159.27 | 121.32 | 137.53 |
| 90 | 207.51 | 216.92 | 139.72 | 153.92 |
| 120 | 247.12 | 256.13 | 147.96 | 165.01 |

Effect of irradiated psoralen on the in vitro glucose oxidation by brain homogenate

Since normal psoralen was found to have no effect on the glucose oxidation, it was considered worth while to see the effect of irradiated psoralen on the *in vitro* glucose oxidation by brain homogenate. The effect of psoralen irradiated for half, one, two, three, four and six hours on brain homogenate has been presented in Table II. The data presented would show that half an hour irradiated psoralen brought about an inhibition of 28.53 per cent after 30 minutes of incubation. There was not much difference in the inhibition of glucose oxidation by half and one hour irradiated psoralen in 30 minutes of incubation, however in later period of incubation, it was observed that the percentage inhibition with half hour irradiated psoralen was almost constant throughout the period of incubation (upto 2 hours), while with one hour irradiated psoralen the percentage inhibition increased from 30.91 per cent to 33.07 per cent.

A constant increase in the inhibition of glucose oxidation was observed with increasing time of irradiation of psoralen, the percentage inhibition brought about by 2,3 and 4 hours irradiated psoralen being 34.78, 40.97 and 43.47 per cent respectively at the 30 minutes incubation period. A uniform pattern was

Table II - Effect of irradiated psoralen on the metabolism of glucose in rat brain homogenate.

| Time (minutes) | Oxygen consumed (µl.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | A | B | C | D | E | F |
| 30 | 85.98 | 61.44 (28.53) | 59.30 (30.91) | 56.07 (34.78) | 50.83 (40.87) | 48.60 (43.47) | 49.23 (42.74) |
| 60 | 154.26 | 110.04 (28.66) | 104.75 (32.10) | 99.21 (35.68) | 88.95 (42.33) | 84.30 (45.46) | 87.45 (43.31) |
| 90 | 207.51 | 147.17 (29.07) | 134.98 (34.95) | 130.06 (37.32) | 115.01 (44.59) | 107.24 (48.32) | 113.72 (45.19) |
| 120 | 247.12 | 173.78 (29.67) | 150.57 (39.07) | 152.35 (38.34) | 133.67 (45.90) | 119.78 (50.13) | 128.86 (47.89) |

The data in the columns A,B,C,D,E and F represent the microlitres of oxygen consumed with half, one, two, three, four and six hours irradiated psoralen respectively.

The values in parenthesis represent the per cent inhibition.

observed with psoralen irradiated for different periods and the inhibition was found to increase with time of incubation. The final percentage of inhibition after two hours of incubation was seen to be 38.34, 45.90 and 50.13 for 2,3 and 4 hours irradiated psoralen respectively. The inhibition brought about by six hours irradiated psoralen was almost the same as that observed with 4 hours irradiated sample. This perhaps may be due to the completion of degradation of psoralen or some of its component (or components) responsible for the inhibition of glucose oxidation.

The average inhibition brought about by psoralen irradiated for different periods has also been calculated and the results are presented in Table III.

Table III - Average inhibition of glucose metabolism of brain homogenate brought about by irradiated psoralen.

| Irradiation time (hours) | Per cent inhibition |
|---|---|
| 0.50 | 28.98 |
| 1.00 | 34.26 |
| 2.00 | 36.53 |
| 3.00 | 43.42 |
| 4.00 | 46.94 |

Here again one finds that the percentage inhibition showed a rise as the period of irradiation was increased (23.93 per cent with half an hour irradiated psoralen and 46.94 per cent for four hours irradiated psoralen). With six hours irradiated psoralen however the value was not found to exceed that of four hours irradiated psoralen.

With a view to have a clearer picture, the results have been presented graphically in Fig. I, from which it is once again clear that oxidation of glucose is inhibited by irradiated psoralen and increases with increase in the time of irradiation upto four hours, and on further irradiation upto six hours does not bring about any further decrease in the oxidation.

<u>In vitro effect of irradiated psoralen on glucose oxidation by rat liver homogenate.</u>

Effect of psoralen irradiated for half, one, two, three, four and six hours was also studied on glucose metabolism of liver and the results are presented in Table IV. The data would show that half an hour irradiated psoralen, in 30 minutes of incubation, brought about an inhibition of 13.47 per cent which dropped to 6.0 per cent after two hours of incubation. With the psoralen irradiated for one, two,

Fig. 1 Inhibition of glucose oxidation by irradiated psoralen in brain homogenate.

A - Control

B,C,D and E represent the oxygen consumed with 0.5, 1.0, 2.0 and 3.0 hours irradiated psoralen respectively.

FIG.1

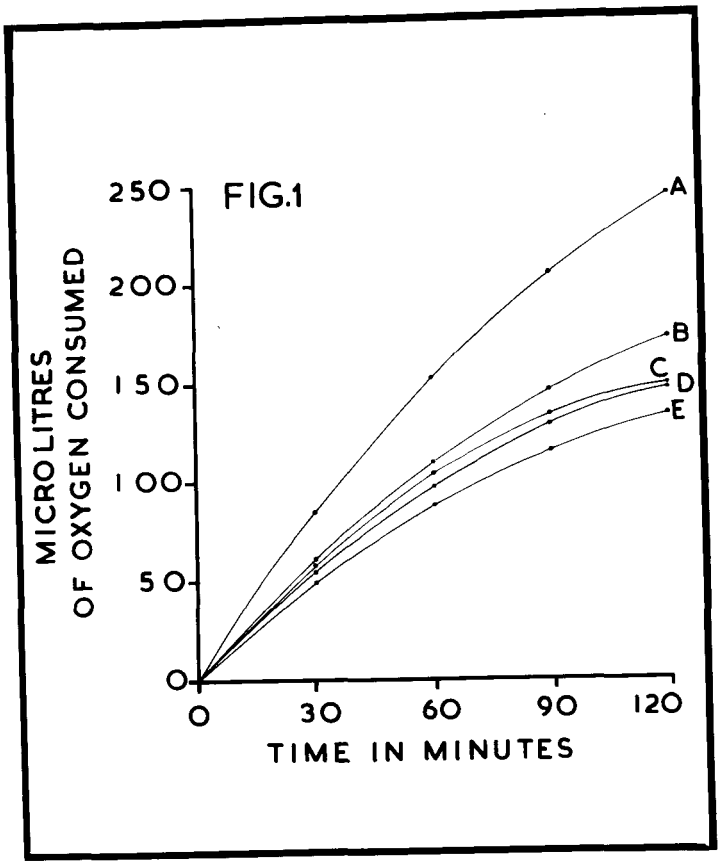

250
200
150
100
50
0
MICROLITRES
OF OXYGEN CONSUMED
0
30
60
90
120
TIME IN MINUTES
A
B
C
D
E

Table IV - Effect of irradiated psoralen on the metabolism of glucose by rat liver homogenate.

| Time (minutes) | Oxygen consumed (µl.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | A | B | C | D | E | F |
| 30 | 86.42 | 74.79 (13.47) | 63.04 (27.05) | 57.24 (33.76) | 49.61 (38.17) | 47.82 (44.66) | 48.27 (44.14) |
| 60 | 121.32 | 106.92 (11.86) | 90.65 (25.28) | 84.17 (30.61) | 76.71 (36.76) | 68.84 (43.25) | 69.09 (43.05) |
| 90 | 139.72 | 128.21 ( 8.18) | 106.00 (24.13) | 99.15 (29.04) | 90.01 (35.57) | 81.22 (41.97) | 81.98 (41.11) |
| 120 | 147.96 | 139.26 ( 6.00) | 113.90 (23.01) | 106.50 (28.03) | 97.17 (34.32) | 86.50 (41.53) | 87.76 (40.68) |

The data in the columns A,B,C,D,E and F represent the microlitres of oxygen consumed with half, one, two, three, four and six hours irradiated psoralen respectively.

The values in parenthesis represent the per cent inhibition.

three, four and six hours the same pattern is also apparent viz. the values which were 27.05, 33.76, 38.17, 44.66 and 44.14 per cent respectively, in 30 minutes of incubation went down to 23.01, 28.03, 34.32, 41.52 and 40.69 per cents after two hours of incubation. It is also clear from the table that the inhibition of glucose oxidation depends upon the period for which psoralen has been irradiated, it being greater with the longer exposure, however it may be said that a more rapid increase in the inactivation was observed in the initial period of irradiation of psoralen upto one hour (13.47 per cent to 27.05 per cent).

The average inhibition of oxidation of glucose in rat liver homogenate has been presented in Table V.

Table V - Average inhibition of glucose metabolism of liver homogenate brought about by irradiated psoralen.

| Irradiation time (hours) | Per cent inhibition |
|---|---|
| 0.50 | 9.88 |
| 1.00 | 24.86 |
| 2.00 | 30.36 |
| 3.00 | 36.20 |
| 4.00 | 42.84 |

The data would show that half an hour irradiation brings about an overall inhibition of 9.88 per cent which increased to 24.86, 30.30, 36.20 and 42.84 per cent when one, two, three and four hours irradiated psoralen was used. The extent of inhibition is more marked when the irradiation of psoralen was increased from half an hour to one hour, after which the inhibition was found to increase slowly but gradually.

Fig. 2 represents the microlitres of oxygen consumed in glucose oxidation by normal liver homogenate and the homogenate with psoralen irradiated for different periods. From the figure it is once again seen that irradiated psoralen brings about an inhibition of the oxidation of glucose and the inhibition is proportional to the time of irradiation of psoralen.

Effect of feeding psoralen on glucose oxidation by rat liver and brain homogenate.

Effect of feeding psoralen on the metabolism of glucose in liver and brain homogenate was studied and the results are presented in Table VI.

Fig. 2 Inhibition of glucose oxidation by irradiated psoralen in liver homogenate.

A - Control

B, C, D and E represent the oxygen consumed with 0.5, 1.0, 2.0 and 3.0 hours irradiated psoralen respectively.

FIG.2

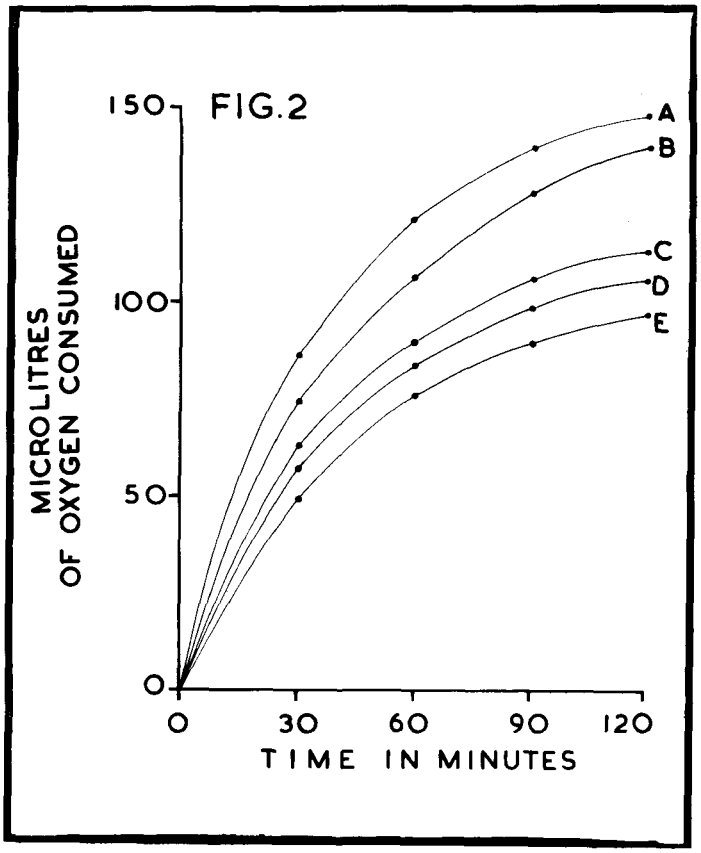

150
100
50
0
MICROLITRES
OF OXYGEN CONSUMED
A
B
C
D
E
0
30
60
90
120
TIME IN MINUTES

Table VI - In vivo glucose oxidation by liver and brain of normal and psoralen fed rats.

| Time (minutes) | Oxygen consumed (µl.) | | | |
|---|---|---|---|---|
| | Brain | | Liver | |
| | Normal | Psoralen fed | Normal | Psoralen fed |
| 30 | 57.00± 2.53 | 57.48± 6.46 | 70.02±20.74 | 54.25±19.73 (22.31) |
| 60 | 101.40± 9.12 | 97.23± 8.31 | 106.15±18.97 | 81.07±16.97 (23.71) |
| 90 | 129.47± 9.33 | 128.89±12.70 | 119.53±26.24 | 92.57±21.37 (22.53) |
| 120 | 153.18±14.13 | 148.60±12.70 | 138.15±23.54 | 105.79±25.45 (23.41) |

The values in parenthesis represent the per cent inhibition.

It is evident from the data presented that feeding of psoralen for a week does not bring about any change in the glucose oxidation of brain while the oxidation of liver was diminished to a considerable extent. The overall average inhibition was found to be 22.91 per cent.

## DISCUSSION

Inspite of the fact that psoralen and other furocoumarins have been commercially used in the treatment of vitiligo and in sun-tanning lotions, very little information is available on their metabolism *per se* or their effect on tissue enzymes. Lerner[2] reported no action of methoxsalen on skin tyrosinase. In the present studies also no action has been obtained by psoralen suspension on the *in vitro* oxidation of glucose in brain and liver homogenates. This would be expected since psoralen as such is known to be almost insoluble at neutral pH. Slight activation, although insignificant, could possibly be due to the very minute impurities carried over during the isolation and purification of this furocoumarin from *Psoralea corylifolia*.

Irradiated psoralen on the other hand was found to be an effective inhibitor of glucose oxidation in both brain and liver homogenates. This is not at all

surprising in view of the inactivation of -SH groups (cf. Chapter III) by irradiated product of psoralen, since many of the enzymes participating in such reactions are known to contain -SH groups[11], essential for their activities. The increase in the inhibition of *in vitro* glucose oxidation is primarily due to the increased formation of the inhibitory products when psoralen was irradiated for longer periods. This is all the more convincingly brought about when almost no further increase in the inhibition was obtained after four hours of irradiation, the percentage inhibition of four and six hours irradiated psoralen being 42.84 and 42.34 with liver while 46.84 and 44.78 with brain respectively.

The *in vivo* studies however revealed inhibition in the glucose oxidation only in liver, brain being almost unaffected. This is in agreement with the clinical studies on psoralen and other furocoumarins which are known to produce liver necrosis[2,8]. The damaged tissue perhaps does not metabolize glucose to the same extent. Resistance of the brain in *in vivo* studies could perhaps be due to the existance of already established blood brain barrier, although according to Griffin[1] significant amount of label from 8-MOP was observed in this organ. This will

probably mean that the concentrations reached in brain are not enough to inhibit the exogenous glucose oxidation.

These studies in a way point out to the *in vitro* and *in vivo* effect of psoralen on the overall carbohydrate metabolism in terms of glucose oxidation in brain and liver tissue. However exact correlation of the significance of these findings with pigment production will have to await further experimentation along these lines with different organs using other substrates. These studies are certainly a pointer to the metabolic inhibition, possibly caused by the administration of furocoumarins in general and psoralen in particular.

## SUMMARY

The effect of normal and irradiated psoralen has been studied in *in vitro* and *in vivo* on the glucose oxidation of brain and liver homogenates. Psoralen *per se* was found to have no effect on the *in vitro* glucose oxidation while irradiated psoralen brought about an inhibition of the glucose oxidation in both liver and brain homogenates. The average inhibition for half, one, two, three and four hours irradiated

psoralen in brain was observed to be 28.38, 34.26, 36.53 and 43.42 per cent respectively. Six hours irradiated psoralen, however, did not give an increased inhibition. In liver homogenate half an hour irradiated psoralen brought about an overall inhibition 9.38 per cent which increased to 24.86, 30.30, 36.20 and 42.84 per cent when psoralen was irradiated for one, two, three and four hours respectively. Six hours irradiated psoralen once again did not bring about an increased inhibition. Feeding of psoralen to albino rats for one week was not found to bring about any change in the glucose oxidation of brain whereas the oxidation of liver was seen to be inhibited significantly.

REFERENCES


1. Griffin, A.C., J.Invest.Dermatol., 32 (1959) 367.
2. Lerner, A.B., Denton, C.R. and Fitzpatrick, T.B., Ibid., 20 (1953) 299
3. Lerner, A.B. and Case, J.D., Ibid., 32 (1959) 211.
4. Kitagawa, H., J.Pharm.Soc.Japan, 74 (1954) 271.
5. Idem Ibid., 75 (1955) 589.

6. Michaelis, M. and Quastel, J.H., — Biochem.J., 35 (1941) 519.

7. El-Mosty, A., El-Mosty, A.M., Abdelal, H. and El-Hawary, M.F.S., — Arch.Dermatol., 90 (1959) 53.

8. Elwi, A.M., — J.Egypt.Med.Assoc., 33 (1950) 773.

9. Mukherji, A., — J.Sci.Indus.Res.(India), 19C (1960) 223.

10. Umbreit, W.W., Burles, R.H. and Stauffer, J.F., — 'Manometric Techniques', Burgess Publishing Co., Minneapolis (1957) p.149.

11. Barron, E.S.G., — 'Thiols Groups of Biological Importance', In Advances in Enzymology, II (1951) 232-244.

# CHAPTER VII

## EFFECT OF FEEDING PSORALEN ON THE COPPER CONTENT OF DIFFERENT ORGANS IN ALBINO RATS.

Copper deficiency in animals has been reported to give rise to greying of hair, and its addition has been found to restore the normal colour[1-4]. Formation of melanin pigment is brought about by tyrosinase action mediated through a number of steps. Copper has been shown to be essential for this enzyme[5-16] and addition of KCN and dialysis is seen to bring about a loss in its activity, which is easily restored by addition of copper ions. Other metals were found to be ineffective. Many thiols, known to be effective inhibitors of tyrosine-tyrosinase reaction, has been shown to combine with the copper moiety of the enzyme, and thereby bring about an inhibition in melanin formation[17]. The valence state of copper in the enzyme, although unknown, is generally believed to be in the cuprous state[18] and is firmly bound to the enzyme protein[19,20].

Contradictory reports are available regarding the serum copper level in various pigmentory disorders. According to Lerner[21] serum copper appears to be of no

significance in vitiligo, although Schuppli[22] has reported an indication of its elevation in such cases. Mofty et al.[23] studied the serum copper in a large number of normal and vitiligo patients and showed a slight decrease in its level in the latter cases. Herrmann et al.[24] studied serum copper in various dermatoses but could not demonstrate any relationship between pathological dermatosis and blood copper concentration. Recently Kandhari and Sobhanadri[25] studying serum copper in pigmentory disorders, observed no significant alteration in its level either in cases of vitiligo or in patients suffering from hyperpigmentation.

Furocoumarins have been known to induce pigmentation in vitiliginous skin when administered along with longwave ultraviolet or solar irradiation[25-28]. The precise mechanism of this induction is at present largely unknown. Mofty et al.[29] have reported that feeding of 8-MOP to albino rats caused a marked rise in blood copper and a significant drop in its content in liver. However 8-isoamyleneoxypsoralen was found to be ineffective. The same workers[30] have further established that pituitary is responsible for the mobilization of copper from liver to blood. Repigmentation of skin in patients treated with 8-MOP or 8-isoamyleneoxypsoralen has also been shown to result

in a significant rise in serum copper[31]. Hence it was considered worthwhile to investigate the effect of feeding psoralen for different periods and observing its effect on total copper of the different organs in albino rats. The result of such studies are reported in the present chapter.

## METHODS AND MATERIALS

### Feeding of rats

Male albino rats of about 100 gm weight taken from Central Drug Research Institute Colony were divided in the following four groups of eight rats each and were housed separately in different cages:-

Group I - Normal

Group II - 3 days psoralen fed

Group III - 7 days psoralen fed

Group IV - 15 days psoralen fed.

To group I, control solution was fed while the rats of other group were, in addition, administered psoralen solution. The composition of each mixture and solution ~~is~~ was as described in Chapter V. After the requisite period, the rats were killed by breaking the spinal cord and the organs were immediately removed. 1-2 gm of the tissue was weighed and transferred to

Kjeldahl flask, to which 5 ml of mixture of sulfuric and nitric acid (3:1) was added and the flasks were then kept for digestion. After the digest became transparent, few drops of hydrogen peroxide were further added to finally decolourise the solution. Polarographic method was used for the estimation of copper.

Reagents

The mercury used in the dropping mercury electrode (D.M.E.) was first purified by chemical treatment with dilute nitric acid, degreased with 0.1N sodium hydroxide, washed, dried and then distilled twice under reduced pressure. Oxygen was removed from the solution in the polarographic cell with a stream of oxygen free nitrogen, purified by bubbling through vanadous sulphate solution. All other chemicals used were of Analar grade and solutions were prepared in glass distilled water.

Apparatus

Polarograms were taken with Lange's manual polarograph using a Multiflex Galvanometer for recording currents. All potentials were measured against a Hume and Harris saturated Calomel electrode. The

temperature was maintained at 37±0.5°C in a thermostatically controlled water bath.

Procedure

The digested samples of various organs containing concentrated sulphuric acid were as such used for polarography. Complete polarograms of the first few samples were taken to ascertain the number of possible reducible compounds in the solution and to guess their identity. Since almost all organic matter is oxidized during digestion only inorganic compounds were considered. A single well defined polarographic wave was observed. In all the samples the half-wave potential ($E_{\frac{1}{2}}$) was exactly at 0 volts (versus S.C.E.). Since this value of the half-wave potential is reported for copper (II) ions in this medium, it was concluded that the polarographic wave was due to copper (II) ions. This was further confirmed by recovery experiments in which the half-wave potential was also found to be 0 volts. The concentration of copper was calculated by measuring the diffusion current at -0.200 volts (versus S.C.E.). The effect of different concentrations of sulphuric acid, different ionic strengths obtained by the addition of potassium chloride and potassium sulphate was also investigated and found to

be negligible. The digested samples of various organs of the rats were polarographed as such without the addition of any other supporting electrolyte.

## RESULTS

Before actually doing the copper content of different organs of normal and psoralen fed albino rats, it was of interest to do some recovery experiments in order to know the error in the estimation. To each digested solution of the organs different amounts of copper was added. At least three such additions were done with different amounts of copper. Percentage error was calculated with all the eight organs under study, namely, liver, kidney, heart, brain, lung, spleen, skin and muscle and the results are presented in Table I. The error varies from ±2 to ±5 per cent in most of the organs, except in spleen and kidney in which few estimations were found to give slightly higher percentage of error. It would be interesting here to mention that according to Page[32], under normal conditions, the accuracy of polarographic analysis is about ±2% in the concentration range of $10^{-2}$ to $10^{-4}$M and ±5% at $10^{-4}$ to $10^{-5}$M, which is quite in agreement with the above results.

Table I - Recovery experiments in different organs of albino rats.

| Organs | Per cent error in set number | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Liver | - 1.2 | + 2.7 | - 2.0 |
| Skin | - 1.7 | - 2.6 | - 1.9 |
| Heart | + 4.4 | + 6.6 | - 5.7 |
| Brain | - 5.1 | - 7.7 | - 6.6 |
| Kidney | - 4.3 | - 8.8 | - 9.9 |
| Lung | - 2.2 | + 1.0 | + 1.6 |
| Spleen | - 6.4 | - 4.3 | - 8.2 |
| Muscle | + 3.1 | + 3.3 | + 4.2 |

Table II represents the total copper in different organs of eight animals. The data presented would show that the copper content of liver was found to vary from 7 to 11 mg per cent. However, two values were in the range of 2 to 5 mg per cent. The average mean value with standard deviation works out to be 8.00 ± 3.27. In all the other organs the pattern of variation was essentially the same and the mean values for skin, heart, brain, kidney, lung, spleen and muscle as indicated in table are seen to be 3.35±2.77, 4.95±1.32, 1.13±0.67, 4.61±2.41, 5.25±3.70, 9.16±4.64 and 2.07±1.59 respectively.

Table II - Total copper in different organs of normal albino rats.

| Rat no. | Copper content of various organs (in mg per cent) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Liver | Skin | Heart | Brain | Kidney | Lung | Spleen | Muscle |
| 1 | 2.56 | 8.09 | 5.02 | 1.23 | - | 13.10 | 14.31 | 3.38 |
| 2 | 6.41 | 2.15 | 5.77 | 1.42 | 6.21 | 3.53 | 5.98 | 0.35 |
| 3 | 10.83 | 2.93 | 7.37 | 1.49 | 6.19 | 7.84 | 8.54 | 1.63 |
| 4 | 11.70 | 1.25 | 3.52 | 0.23 | - | 0.76 | 15.53 | 5.63 |
| 5 | 9.02 | 0.63 | 3.65 | 1.22 | 5.17 | 4.97 | 9.64 | 0.96 |
| 6 | 10.58 | 6.75 | - | 1.20 | 6.58 | 6.50 | - | 1.82 |
| 7 | 8.48 | - | - | 2.37 | 3.08 | 3.90 | 9.66 | 1.06 |
| 8 | 4.40 | 1.63 | - | 0.35 | 0.42 | 1.30 | 0.60 | 1.74 |
| Mean | 8.00 | 3.35 | 4.95 | 1.18 | 4.61 | 5.25 | 9.16 | 2.07 |
| ± S.D. | 3.27 | 2.77 | 1.32 | 0.67 | 2.41 | 3.70 | 4.64 | 1.59 |

Table III, IV and V represent the total copper in different organs of psoralen fed rats for the 3,7 and 15 days respectively. A comparison of the data in the different groups would indicate that:-

(a) Copper content of the spleen indicated a rise of around 46.20% (9.16±4.64 to 13.40±2.58 mg per cent) after 3 days of psoralen administration and this increase was more or less maintained even after 15 days of psoralen feeding.

(b) Liver on the other hand exhibited a decrease in its copper content by about 42.87% after the first 3 days of psoralen administration and this was maintained upto 15 days.

(c) Skin was found to give a variable response at different periods. Initial feeding for 3 days indicated a rise of about 43.52% (3.35 to 4.81 mg%). Further feeding for 7 days however was found to bring about a decline in the copper content to 1.40 mg per cent. On continued feeding for 15 days, the normal value of 3.25 mg% was obtained.

(d) The other organs, brain, heart, kidney, lung and muscle did not indicate any significant difference in

Table III - Total copper in different organs of psoralen fed ( 3 days) albino rats.

| Rat no. | Copper content of various organs (in mg per cent) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Liver | Skin | Heart | Brain | Kidney | Lung | Spleen | Muscle |
| 1 | 4.41 | 4.35 | - | 2.07 | 1.00 | - | - | 1.04 |
| 2 | 6.92 | 2.95 | 4.83 | 0.46 | 2.14 | 3.46 | 10.00 | 3.40 |
| 3 | 7.01 | 6.20 | - | 2.04 | 2.94 | 3.82 | 14.97 | 2.52 |
| 4 | 7.71 | 10.02 | - | 0.65 | 3.64 | 8.20 | 11.42 | 1.47 |
| 5 | 7.71 | - | - | 1.09 | 2.83 | 8.87 | - | 4.11 |
| 6 | 0.93 | 6.18 | 11.28 | 2.35 | 5.20 | 7.40 | 12.82 | 2.25 |
| 7 | 0.69 | 0.75 | 3.18 | 3.00 | 3.58 | 3.93 | 13.95 | 2.85 |
| 8 | 0.64 | 3.22 | 1.55 | 0.97 | 3.81 | 1.25 | 17.23 | 1.09 |
| Mean | 4.50 | 4.81 | 5.22 | 1.58 | 3.14 | 5.27 | 13.40 | 2.29 |
| ± S.D. | 3.27 | 2.77 | 4.36 | 0.90 | 1.16 | 2.87 | 2.58 | 1.07 |

Table IV - Total copper in different organs of psoralen fed (7 days) albino rats.

| Rat no. | Copper content of various organs (in mg per cent) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Liver | Skin | Heart | Brain | Kidney | Lung | Spleen | Muscle |
| 1 | 5.10 | 4.18 | - | 1.45 | - | - | 8.57 | 1.46 |
| 2 | 6.60 | 0.48 | 3.96 | 1.30 | 3.36 | 2.26 | 13.00 | 3.44 |
| 3 | 5.00 | 1.05 | 5.40 | 1.37 | 6.63 | 7.60 | 17.69 | 2.11 |
| 4 | 3.52 | 0.86 | - | 0.78 | 3.17 | 3.65 | 9.01 | 1.03 |
| 5 | 6.57 | 0.78 | 1.88 | 0.14 | 0.40 | 11.58 | 21.40 | 1.41 |
| 6 | 1.47 | 1.33 | 10.63 | - | 4.75 | 1.45 | 0.96 | 0.89 |
| 7 | 3.78 | 1.11 | 7.78 | 0.84 | 0.44 | 3.67 | 4.07 | 4.28 |
| 8 | 6.00 | - | 3.56 | 1.88 | 4.19 | 3.20 | 23.22 | 1.56 |
| Mean | 4.75 | 1.40 | 5.47 | 1.09 | 3.28 | 4.77 | 12.24 | 2.02 |
| ± S.D. | 1.45 | 1.29 | 3.38 | 0.56 | 2.47 | 3.57 | 8.05 | 1.32 |

Table V - Total copper in different organs of psoralen fed (15 days) albino rats.

| Rat no. | Copper content of various organs (in mg per cent) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Liver | Skin | Heart | Brain | Kidney | Lung | Spleen | Muscle |
| 1 | 2.26 | - | 6.30 | 2.54 | 3.77 | 4.43 | 21.78 | 2.10 |
| 2 | 5.00 | 4.29 | - | 2.37 | - | 3.33 | 5.91 | 1.52 |
| 3 | 6.32 | 4.60 | 5.41 | 4.89 | 3.91 | - | - | 1.71 |
| 4 | 4.24 | 0.50 | 16.54 | 0.99 | 2.32 | 2.58 | 15.19 | 2.47 |
| 5 | 3.83 | 4.17 | - | 2.13 | 3.85 | 5.78 | 13.70 | 2.38 |
| 6 | 2.85 | 4.90 | 2.59 | 0.87 | 2.99 | - | - | 1.90 |
| 7 | 3.63 | 0.42 | 4.18 | 3.09 | 3.34 | 8.56 | 12.59 | 3.95 |
| 8 | 7.14 | 3.90 | 3.49 | 1.89 | 4.66 | 7.93 | 14.90 | 6.27 |
| Mean | 4.46 | 3.25 | 6.42 | 2.34 | 3.48 | 5.43 | 13.99 | 2.79 |
| ± S.D. | 1.67 | 1.84 | 2.46 | 1.27 | 0.75 | 2.71 | 5.10 | 2.32 |

Fig. 1 *In vivo* effect of feeding psoralen on the total copper content of brain, spleen, liver and skin.

1 - Spleen

2 - Liver

3 - Skin

4 - Brain

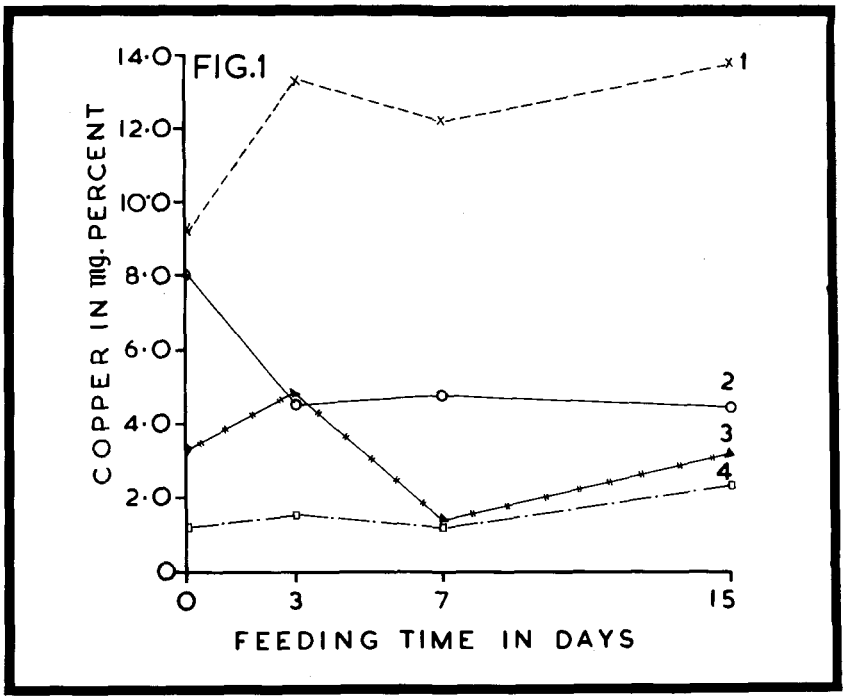


FIG.1
14·0
12·0
10·0
8·0
6·0
4·0
2·0
0
COPPER IN mg. PERCENT
0
3
7
15
FEEDING TIME IN DAYS
1
2
3
4

Fig. 2 *In vivo* effect of feeding psoralen on the total copper content of heart, kidney, lung and muscle.

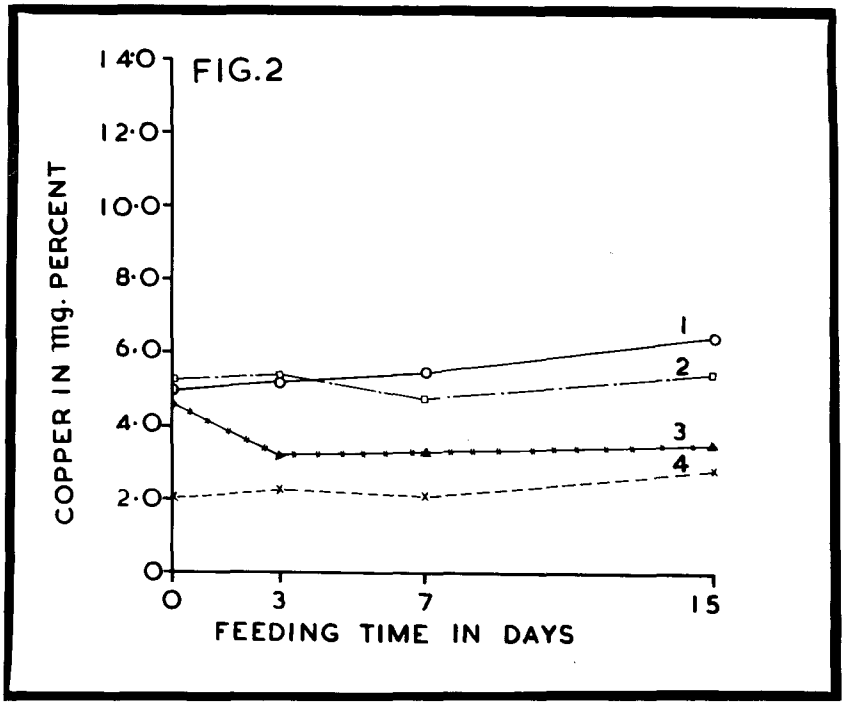
1 - Heart

2- Lung

3 - Kidney

4 - Muscle

FIG.2


14·0
12·0
10·0
8·0
6·0
4·0
2·0
0
COPPER IN mg. PERCENT
0
3
7
15
FEEDING TIME IN DAYS
1
2
3
4

the copper content of normal and psoralen fed rats for the period 3,7 and 15 days.

With a view to have a better comprehension, the average copper content of different organs of normal and psoralen fed rats has been presented graphically in Fig. 1 and 2. Once again it is evident from the figures that the copper content of heart, kidney, lung, muscle and brain is not changed by feeding psoralen for 3,7 and 15 days. However, in spleen, liver and skin psoralen administration brought about a significant variation.

## DISCUSSION

The result of the present study would show that feeding of psoralen for the periods, 3,7 and 15 days brought about a significant decrease of the copper content in liver. These results are in close agreement with the observation of Hefty et al.[29] who reported a marked rise in blood copper and a drop in liver after the administration of 8-methoxypsoralen. They have further established that pituitary is responsible for the mobilization of copper from liver to blood[30]. Repigmentation of skin in patients treated with 8-methoxypsoralen or 8-isoamyloxypsoralen has also been

shown to result in a significant rise in serum copper[31]. It would thus appear that in view of the requirement of copper at other sites, psoralen administration depletes copper from the liver, giving rise to its increase in the peripheral blood circulation.

Having accepted that psoralen induces copper to migrate from liver to peripheral blood, its increase in spleen could probably be explained on the basis of spleen being a blood filter. Copper probably is bound to such proteins in the circulatory blood that are easily permitted by spleen to pass through.

The initial increase of copper in the skin in the first three days of psoralen supplementation could possibly be due to the uptake of copper from the peripheral blood stream. In albino skin, this increase in copper did not bring about any material advantage and therefore once again was excreted into the blood stream and the values were found to return to the normal. The decrease after seven days of psoralen feeding may possibly point out to some sort of excretory mechanism which may have been developed in this tissue, after the accumulated copper was not required.

The inertness in other organs like brain, heart, kidney, lung and muscle would perhaps indicate the possible insignificance of such organs in the action of psoralen in pigment production. It would thus appear

from these studies that one of the mechanism by which psoralen may exert its action in the production of melanin, may be that it somehow (exact mechanism for this process is still unknown) releases the stored copper from the liver and through peripheral blood circulation, makes it available to the depleted vitiliginous areas. However this hypothesis will have to await further confirmation by the use of radioactive copper and finally clinical studies in human beings.

## SUMMARY

A comparison of the total copper content in different organs of the psoralen fed (3,7 and 15 days) rats would indicate that its content in spleen is increased by about 46.30 per cent. while that of liver exhibited a decrease of around 42.87 per cent after three days of psoralen administration. The values were essentially the same even after 15 days of psoralen feeding. Skin however, was found to have a variable response at different periods. Initial feeding for 3 days indicated a rise of about 43.52 per cent. Further feeding for 7 days however was found

to bring about a decline in its copper content. On continued feeding for 15 days, normal values were again obtained. The other organs, brain, heart, kidney, lung and muscle did not indicate any significant differences in their copper content. The possible significance of these changes has been discussed *vis-a-vis* the role of psoralen in pigment production.

REFERENCES


1. Keil, H.L. and Nelson, V.E., J.Biol.Chem., 93 (1931) 49.
2. Carter, F.J., Nature, 136 (1935) 185.
3. Sjollema, B., Biochem.Z., 295 (1938) 372.
4. Smith, S.W. and Ellis, G.H., Arch.Biochem., 15 (1947) 81.
5. Kubowitz, F., Biochem.Z., 292, (1937) 221.
6. *idem* ibid., 293 (1938) 32.
7. Allen, T.H. and Bodine, J.H., Science, 94 (1941) 443.
8. Ludwig, H.J. and Nelson, J.M., J.Am.Chem.Soc., 61 (1939) 2601.
9. Keilin, D. and Mann, T., Proc.Roy.Soc.London,Ser.B., 125 (1938) 187.
10. Parkinson, G.C.(Jr) and Nelson, J.M., J.Am.Chem.Soc., 62 (1940) 1693.

11. Dalton, H.R. and Nelson, J.M., — J.Am.Chem.Soc., 61 (1939) 2946.

12. Sreerangachar, H.B., — Biochem.J., 37 (1944) 667.

13. Lerner, A.B., Fitzpatrick, T.B., Calkins, E. and Summerson, W.H., — J.Biol.Chem., 187 (1950) 793.

14. Lerner, A.B., — Arch.Biochem.Biophys., 36 (1952) 473.

15. Kertesz, D., — Biochim.Biophys.Acta, 9 (1952) 170.

16. Polonovski, M. and Gonnard, P., — Compt.Rend.Soc.Biol., 145 (1957) 1612.

17. Rothman, S., — 'Physiology and Biochemistry of skin'. The University of Chicago Press. Chicago (1950) pp.531-533.

18. Kertesz, D., — Nature, 180 (1957) 506.

19. Dressler, H. and Dawson, C.R., — Biochim.Biophys.Acta, 45 (1961) 508.

20. Idem — Ibid., 45 (1961) 515.

21. Lerner, A.B., — J.Invest.Dermatol., 32 (1959) 285.

22. Von Schuppli, R., — Dermatologica, 104 (1952) 231.

23. El-Mofty, A.M., El-Hawary, M.F.S. and Farag, F.B., — J.Egypt.Med.Assoc., 44 (1961) 13.

24. Hermann, R., Kafka, J. and Knoth, W., — Dermatol.Wochenschri., 142 (1960) 1265.

25. Kandhari, K.C. and Sobhanadri, C., — Indian J.Dermatol.Venereol., 29 (1963) 141.

26. El-Mofty, A.M., J.Roy.Egypt.Med.Assoc., 31 (1948) 651.

27. Lerner, A.B., Denton, C.R. and Fitzpatrick, T.B., J.Invest.Dermatol., 20 (1953) 299.

28. Fitzpatrick, T.B., Hopkins, C.E., Blickenstaff, D.D. and Swift, S., Ibid., 25 (1955) 197.

29. El-Mofty, A., El-Mofty, A.M., Abdelal, H. and El-Hawary, M.F.S., J.Invest.Dermatol., 32 (1959) 645.

30. Idem Ibid., 32 (1959) 651.

31. El-Mofty, A.M., El-Hawary, M.F.S. and Farag, F.B., J.Egypt.Med.Assoc., 44 (1961) 124.

32. Page, J.E., Quart.Rev.(London), 6 (1952) 262.

# CHAPTER VIII

## GENERAL SUMMARY AND CONCLUSIONS

The work presented in this thesis embodies the work done on a few aspects of "Mechanism of action of psoralen and ultraviolet radiation in pigment production". The details of the conclusion arrived at, although given in the respective chapters, some of the salient points arrived at are as under:-

1. Irradiation of psoralen, both by ultraviolet and solar light, has been found to produce newer fluorescent products having different $R_f$ values. The major product with ultraviolet irradiation was seen to have an $R_f$ of 0.62-0.67 and its absorption spectra, in the ultraviolet region, revealed it to be different from either furocoumaric acid or the dimer of psoralen. The product exhibited a generalized absorption getting stronger towards the shorter wavelength region. Solar irradiation likewise gave rise to two major products, one having bluish fluorescence ($R_f$ 0.36) and the other having greenish bright fluorescence ($R_f$ 0.43). Here again the absorption spectra of the eluates indicated a

generalized absorption, very similar to that of the product obtained with ultraviolet irradiation.

The formation of newer products from psoralen as a result of ultraviolet and solar irradiation appears to be interesting in view of the fact that in the treatment of vitiligo, furocoumarins have been reported to be inactive by themselves unless aided by exposure to ultraviolet or solar light. It may be that one of the transformed products of psoralen may be responsible for accelerating melanin formation.

2. Irradiated psoralen has been found to significantly inhibit the succinic oxidase (an -SH containing enzyme) of rat kidney homogenate, whereas no such effect has been observed with psoralen per se, either in solution or as suspension. Irradiated psoralen was further seen to inactivate thiourea, and thereby reversing its inhibition on potato tyrosinase. In both these studies the -SH inactivating effect was significantly increased when psoralen was irradiated for longer periods. On the basis of this it has been theorized that irradiated psoralen perhaps is able to bring about increased pigmentation by the inactivation of -SH groups, which are known to be important in vivo determinants of tyrosinase activity.

3. Photo-oxidation of dopa by sunlight, white light and ultraviolet light has been shown to be stimulated significantly by the presence of psoralen. This stimulation was seen to be counteracted by the presence of certain -SH compounds as well as when ascorbic acid was incorporated in the reaction mixture. The mechanism by which psoralen may stimulate the pigment production could possibly be due to its accumulation in the melanocytes where it would photo-oxidize the available dopa to melanin, giving rise to increased melanin formation in vitiligo patients.

4. Feeding of psoralen to rats for over two months (with and without ultraviolet radiation) was seen to have almost no effect on their growth. Although the weight of kidney, heart and brain was unaffected by psoralen administration, the weight of spleen was significantly enhanced. Surprisingly enough in animals fed with psoralen with subsequent irradiation, the weight of all the four organs including spleen was found to be unchanged. No significant change was observed by psoralen feeding on the incorporation of radioactive phosphorus in different organs of rats.

5. In *in vitro* experiments psoralen *per se* was found to have no effect on the glucose oxidation by brain and liver homogenate, whereas irradiated psoralen was found to bring about considerable inhibition of glucose oxidation in both the homogenates. Feeding of psoralen to albino rats for a week was found to have no effect on the glucose oxidation of brain homogenate, whereas the oxidation by liver homogenate was considerably diminished.

6. Determination of total copper after 3,7 and 15 days of psoralen feeding, spleen indicated a rise of around 46.20 per cent after three days, liver, on the other hand, exhibited a decrease in its copper content by about 42.87 per cent. This increase in spleen and decrease in liver was found to be maintained even after 15 days of psoralen administration. With skin, however, variable response was obtained. After 3 days of psoralen feeding copper content was found to rise by about 43.52 per cent. On further feeding for 7 days, the copper content actually was observed to decline and normal values were once again attained after 15 days

feeding. Other organs like ~~liver~~, brain, heart, kidney, lung and muscle did not show any appreciable difference in their copper content.

This dissertation would therefore point out to the possible role of psoralen in pigment production. Initially under ultraviolet or solar irradiation its molecules perhaps undergo a change and the transformed product is capable of inactivating the -SH groups and thereby activating tyrosinase. Copper also is depleted by this furocoumarin, through a yet unknown mechanism, from liver and is perhaps taken to the site of vitiliginous areas, through the peripheral blood.

* * * * *

* * *

*